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## THE ANTISPASMODIC ACTION OF BASIC NITRILES

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Previous research devoted to the development of synthetic substitutes for atropine has, for the most part, dealt with basic esters of aryl-aliphatic acids such as 'Trasentine' and 'Pavatrine' or with tropinol esters such as homatropine. More recently, Becker *et al.* (1) have described a series of substituted phenyl-propylpiperidine antispasmodic drugs with comparatively low anticholinergic spasmolytic potency although many of them equalled or exceeded papaverine in their spasmolytic action against barium chloride induced contractures. In an extension of the aforesaid research, a series of 2,2-diphenylaminoalkanenitriles, listed in table 1, has been synthesized by Drs. B. Elpern and A. A. Larsen. This communication describes the results obtained in investigating the pharmacologic properties of these compounds, particularly with regard to their anticholinergic effects.

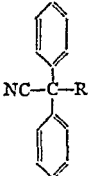


**RESULTS.** 1. *Action on the isolated intestinal segment.* Spasmolytic potencies have been determined by the method of Miller, Becker and Tainter (2) using segments of ileum from both rabbits and guinea pigs. Contractures were induced in the former by acetylcholine and barium and in the latter by histamine. The structural formulas and effective spasmolytic doses, expressed as the log of the weight/volume dilution, are shown in table 1. Examination of these data indicates that small anticholinergic effects are obtained with the tertiary amine hydrochlorides, their relative potencies varying from 0.4 (WIN 1038) to 4.9 (WIN 1601) per cent of that of atropine. Most of the quaternary methiodide salts described here are distinctly more anticholinergic in action than the corresponding tertiary amine hydrochlorides. This increase in potency was greatest for WIN 1038, WIN 1189 and WIN 1185, the methiodide salts being 52, 41 and 35 times more spasmolytic than the corresponding hydrochloride salts, when compared on the basis of their molar concentrations. WIN 1423 and its methiodide (WIN 2246) showed no significant difference in activity. The substitution of a methyl group on the second carbon from the nitrogen appears to be unfavorable for anticholinergic action. WIN 1423 was most spasmolytic against barium chloride contractures, being 24 times more active than papaverine hydrochloride. The conversion of the tertiary amine into the corresponding quaternary methiodide had no predictable effect on the spasmolytic potency against barium contractures. None of the nitriles used in this investigation were found to have important spasmolytic action against histamine induced contractures of the isolated ileal segments of guinea pigs. The quaternary methiodides were found to be distinctly less antihistaminic than the corresponding tertiary amine hydrochlorides.

<sup>1</sup> Deceased.

2. *Action on the intestine in situ.* Spasmolytic action on the small intestine *in situ* was determined in rabbits and dogs by the method of Barbour as described by Jackson (3). In some experiments on rabbits, pilocarpine (1 mgm./kgm.) was injected subcutaneously to increase intestinal tonus and motility. Three of the most active spasmolytic compounds from table 1, WIN 1075, WIN 1234 and WIN 1274, were investigated. Results obtained are shown in table 2. These substances are of comparable potency and cause distinct spasmolysis when injected intravenously in doses of 0.01 mgm./kgm. This is illus-

TABLE 1

A comparison of the spasmolytic action of various diphenylalkanenitrile hydrochlorides with their corresponding methiodide salts

DRUG NO. WIN	 STRUCTURE R	SALT	SPASMOLYTIC POTENCY				ACUTE TOXICITY
			Acetylcholine		BaCl <sub>2</sub>	His-tamine	i.v. mice LD <sub>50</sub> ± s.e.
			Log dilution*	Relative potency†	Log dilution*	Log dilution*	
							mgm./kgm.
1166	—CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	HCl	6.00*	0.8	5.66*	5.74*	33.0 ± 1.5
1193	"	CH <sub>3</sub> I	6.40	2.5	5.76	4.60	13.6 ± 1.2
1038	—CH <sub>2</sub> CH <sub>2</sub> N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	HCl	5.72	0.4	5.90	5.36	34.6 ± 2.5
1075	"	CH <sub>3</sub> I	7.22	21.0	4.48	4.90	7.6 ± 0.3
1185	—CH <sub>2</sub> CH <sub>2</sub> N 	HCl	6.04	0.8	5.94	5.68	26.3 ± 1.2
1234	"	CH <sub>3</sub> I	7.48	28.2	6.40	5.26	4.9 ± 0.4
1601	—CH <sub>2</sub> CH(CH <sub>3</sub> )N(CH <sub>3</sub> ) <sub>2</sub>	HCl	6.72	4.0	6.76	5.96	
1263	"	CH <sub>3</sub> I	7.40	24.4	5.24	<5.00	11.1 ± 0.8
1423	—CH(CH <sub>3</sub> )CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	HCl	6.40	1.9	6.58	5.00	60.0 ± 3.0
2246	"	CH <sub>3</sub> I	6.20	1.5			
1167	—CH <sub>2</sub> CH(CH <sub>3</sub> )N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	HCl	6.08	.7	5.76	5.06	43.7 ± 2.6
1359	"	CH <sub>3</sub> I	6.34	2.0	<6.00	<6.00	5.6†
1169	—CH <sub>2</sub> CH(CH <sub>3</sub> )N 	HCl	6.26	1.3	6.41	5.44	31.0 ± 2.6
1274	"	CH <sub>3</sub> I	7.76	53.0	<6.00	5.30	5.0†
Trasentine		HCl	6.50	2.4	6.50	5.40	43.0 ± 1.0
Homatropine		HBr	6.98	8.4			107.0 ± 7.0
Atropine		H <sub>2</sub> SO <sub>4</sub>	8.10	100.0			75.2 ± 2.3

\* Log of the weight/volume dilution (2).

† Molar concentration of the effective dose of atropine base equals 100 per cent. The molar concentration of all other drugs, as their bases, expressed in per cent concentration of atropine. The molecular weight of the quaternary bases taken as the total molecular weight less the weight of HI.

‡ Approximate LD<sub>50</sub>.

trated by fig. 1, wherein an intravenous injection of 0.01 mgm./kgm. of WIN 1075 produced marked spasmolysis with some effect being apparent for more than 40 minutes. Spasmolytic action on the dog colon was measured in the un-anesthetized animal, the usual condom balloon and tambour system being used to record motility changes. Pilocarpine (0.2 mgm./kgm. intramuscularly) and morphine sulfate (8 mgm./kgm. subcutaneously) were administered to induce hypermotility and the antispasmodic drug was given only after good motility had been established. The effect on the motility of the rabbit colon *in situ* was

TABLE 2  
Spasmolytic effect on the small intestine in situ

ANIMAL*	DRUG	DOSE— $\mu$ g	EFFECT†	DURATION	NUMBER OF EXPERIMENTS
Rabbit	WIN 1075	mgm /kgm		min	
		0.01	+++	10	9
		0.05	+++	10-20	6
		0.10	++++	>25	4
	WIN 1234	0.01	++	Not determined	2
		0.05	+++	10-20	6
	WIN 1274	0.01	0, ++	4-10	8
		0.05	++	8-18	2
		0.10	+, +++	7-10	9
Dog	WIN 1075	0.01	++++	8-15	15
	WIN 1234	0.01	0, ++++	Not determined	8

\* Urethane used as the anesthetic in rabbits. Dogs were anesthetized with sodium pentothal and sodium barbital.

† +++++ indicates a complete abolition of motility with a reduction in tonus.

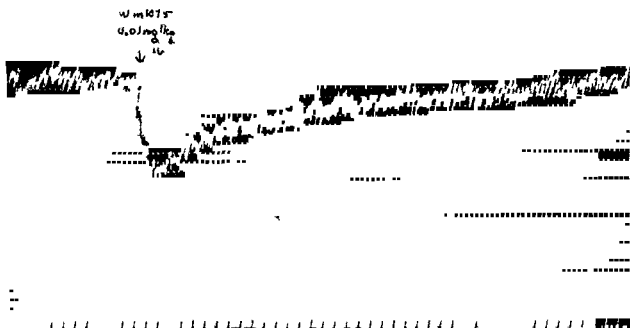


FIG. 1. Dog wt 9.5 kgm. Motility of the small intestine by the method of Barbour. WIN 1075, 0.01 mgm/kgm injected intravenously. Time in minutes.

determined as described above for the rabbit small intestine. Pilocarpine, 1 mgm/kgm, was injected subcutaneously to induce hypermotility. Results obtained with WIN 1075 may be taken as representative of those seen with the most active spasmolytic drugs (table 3). Definite spasmolytic effects on the

rabbit colon were obtained with intravenous doses of 0.01 mgm./kgm. Prolonged spasmolysis of the dog colon was obtained with intramuscular doses of 0.05 mgm./kgm. Oral or sublingual doses of 5-10 mgm./kgm. were effective

TABLE 3  
*Spasmolytic effect of WIN 1075 on the colon in situ*

ANIMAL	DOSE	MODE OF ADMIN	EFFECT	DURATION	NUMBER OF EXPERIMENTS
	<i>mgm /kgm</i>			<i>min</i>	
Rabbit*	0.01	i.v.	++	3-5	4
	0.05	i.v.	+++	15	6
	0.10	i.v.	++++		6
Dog†	0.05	i m.	++++	>35	6
	10.00	Sublingual	++++	>72	1
	5.00	Oral	±, +++	150	2
	10.00	Oral	++++	>60	2

\* Urethane (0.8-1.0 mgm./kgm. s.c.) used as the anesthetic.

† Unanesthetized dog. Hypermotility induced by pilocarpine nitrate (0.2 mgm./kgm.) and morphine sulfate (8.0 mgm./kgm.) subcutaneously.

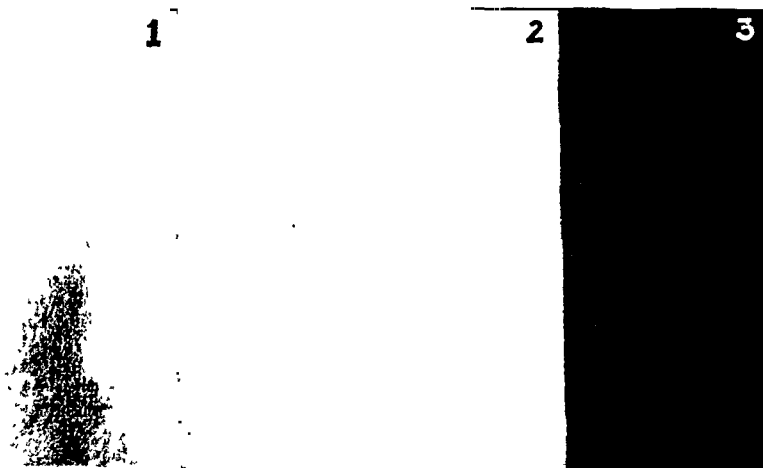


FIG. 2. Dog wt 90 kgm. Unanesthetized but pretreated with morphine sulfate, 8.0 mgm./kgm. subcutaneously and pilocarpine nitrate, 0.2 mgm./kgm. intramuscularly. Effect on colonic motility at an oral dose of 10 mgm./kgm. of WIN 1075. Ten minutes elapsed between 1 and 2, 20 minutes between 2 and 3. Time in minutes.

and results obtained are illustrated in figs. 2 and 3. WIN 1075, like atropine sulfate, is effective when administered orally, sublingually or parenterally.

3. *Effect on salivary secretion.* The effect on salivary secretion was determined in urethanized rabbits. The anticholinergic drug was administered subcutaneously 15 minutes before a subcutaneous injection of 20 mgm./kgm. of pilocarpine nitrate. The saliva secreted was collected, measured and compared

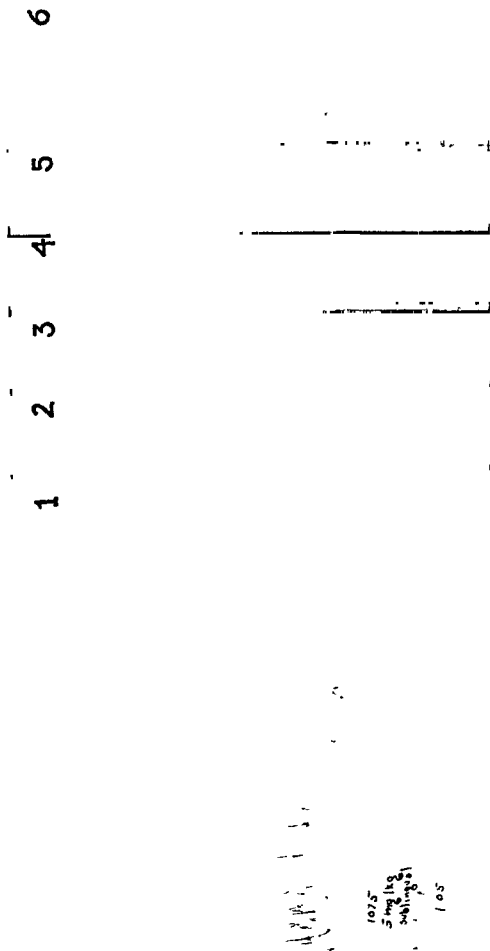


Fig 3. Dog wt 11.6 kgm. Unanesthetized but pretreated with morphine sulfate, 8.0 mgm/kgm, subcutaneously and pilocarpine nitrate, 0.2 mgm/kgm intramuscularly. Effect on colonic motility of a sublingual dose of 5 mgm/kgm of WIN 1075. Ten minutes elapsed between 1 and 2, 20 minutes between each successive segment



with the volume secreted by a comparable group of rabbits injected with pilocarpine alone. The results obtained are shown in table 4. WIN 1075 appears to equal atropine sulfate in its inhibitory effects on salivary secretion. WIN 1234 was somewhat less and WIN 1274 was definitely less effective in blocking secretion.

4. *Vascular anticholinergic effect.* The blocking action against the vaso-depressor effect of acetylcholine was determined in dogs. An amount of acetyl-

TABLE 4  
*Effect on salivary secretion in rabbits*

DRUG	DOSE	EFFECT ON SALIVARY SECRETION,* PER CENT INHIBITION	NUMBER OF EXPERIMENTS
	<i>mgm./kgm.</i>		
WIN 1075	0.1	79	5
	0.5	100	5
	1.0	100	7
WIN 1234	0.1	49	5
	0.5	86	5
	1.0	74	5
WIN 1274	0.1	0	2
	0.5	42	5
	1.0	87	5
Atropine sulfate	0.1	44	3
	0.3	47	4
	0.5	93	4

\* Urethane (1.0 mgm./kgm. subcutaneously) was used as the anesthetic. Salivation induced by pilocarpine (20 mgm./kgm. subcutaneously).

TABLE 5  
*Effect on acetylcholine induced vasodepression in anesthetized dogs*

DRUG	DOSE	REDUCTION IN RESPONSE	DURATION OF EFFECT	NUMBER OF EXPERIMENTS
	<i>mgm./kgm.</i>	<i>per cent</i>	<i>min.</i>	
WIN 1075.....	0.1	57	25-60	4
WIN 1274.....	0.1	58	25->60	3
Homatropine hydrobromide.....	0.1	36	20-30	3
Atropine sulfate.....	0.01	83	35-120	3

choline bromide to produce a 40 to 60 mm. fall in mean carotid blood pressure was injected intravenously and the resulting response was recorded kymographically. The anticholinergic drug was then injected intravenously and the reduction in the vasodepressor response to acetylcholine determined at intervals of 5-10 minutes in order to determine the maximum blocking effect along with the approximate duration of some effect. Results obtained with WIN 1075 and WIN 1274, compared with atropine and homatropine, are shown in table 5.

These synthetic drugs were approximately equal to homatropine but less effective than atropine in blocking the vasodepressor effect of acetylcholine.

5. *Mydriatic effect.* Mydriasis was determined in cats. All drugs were dissolved in distilled water and instilled directly into the conjunctival sac, the eye being held closed for one minute following instillation. Upon release of the eyelids, the excess drug was allowed to drain away. The size of the pupil was determined under direct illumination with a 100 watt electric lamp as the light source. The mydriatic effect was rated from 1+ to 4+, the former representing just perceptible and the latter complete mydriasis with no response to illumination. Results obtained are shown in table 6. Both WIN 1075 and WIN 1234 appear to be slightly more mydriatic than homatropine and distinctly less

TABLE 6  
*Mydriatic effect of anticholinergic drugs in the cat*

DRUG	CONCENTRATION	MYDRIASIS	DURATION OF SOME MYDRIASIS
	<i>per cent</i>		<i>hours</i>
WIN 1075	0.025	++	1-4
	0.050	++	3-6
	0.100	++++	>5
WIN 1234	0.010	++	1-1½
	0.025	+++	2-3
	0.050	+++	3->6
	0.100	++++	>5
WIN 1274	0.50	0, +	
	0.100	0, ++	3->6
	0.250	++++	>6
Homatropine	0.050	+	2½-3½
	0.100	+++	>6
Atropine sulfate	0.001	0, ++	2½
	0.005	+++	>4½
	0.010	++++	>6

mydriatic than atropine. WIN 1274 appears to be slightly less potent than homatropine. By comparison, anticholinergic potency determinations on isolated intestinal segments of rabbit ileum indicate that all three compounds are distinctly more potent than homatropine. Ing *et al.* (4) have suggested that metho-salts, upon local application, may appear less active than similar tertiary bases due to the slowness with which onium cations penetrate cell membranes. Mydriatic potency, as measured here, is influenced by the rate at which the drug passes through the cornea and into the aqueous humor. This may account for discrepancy in results obtained by these two methods.

6. *Effect on the central nervous system.* The stimulating effect of WIN 1075 and WIN 1234 on the central nervous system of albino rats was determined by the method of Schulte, Tainter and Dille (5). Subcutaneous doses of 10-20

mgm./kgm. did not elicit any increase in activity. In other experiments, WIN 1075 was injected intraperitoneally into unanesthetized cats. Doses of 5-20 mgm./kgm. resulted in no demonstrable symptoms of restlessness or incoordination. At a dose of 40 mgm./kgm. some signs of central stimulation and motor incoordination were noted with two out of three injected cats dying within four hours.

7. *Toxicity.* Acute toxicity was determined by intravenous injection into albino mice. The  $LD_{50}$  data calculated by the method of Miller and Tainter (13) are shown in table 1. The quaternary methiodide salts are 2.4 to 7.8 times more toxic than the corresponding tertiary amine hydrochlorides. Oral toxicity in albino mice was determined for WIN 1075 and WIN 1234. By contrast with the results obtained by intravenous administration, both substances were found to have low toxicity (WIN 1075— $1625 \pm 105$  mgm./kgm.; WIN 1234—approximately 800 mgm./kgm.) by this route. These data suggest that absorption from the intestine was slow or that detoxification prevented more than a small amount of the administered salt from reaching the general circulation.

DISCUSSION. Becker *et al.* (1) have reported that the simplest diaryl piperidine propane, 1,1-diphenyl-3-N-piperidylpropane hydrochloride (WIN 118), has 0.66 per cent of the activity of atropine sulfate against acetylcholine induced spasm of the isolated rabbit ileum. The corresponding nitrile analogue (WIN 1185) has comparable activity. The methiodide salt of WIN 118 is approximately twice as potent as the tertiary amine hydrochloride. Examination of the data in table 1 discloses that some methiodide salts are as much as 52 times as active as the corresponding hydrochloride salts. The nitrile group increased significantly the atropine-like action in these quaternary salts. The structure of the substituents on the amine is important inasmuch as with both the propane- and butanenitriles, the N-piperidyl-derivative was distinctly more active than the corresponding dimethyl- or diethylamine derivative.

The position of the methyl group in the isobutylnitriles appears important for spasmolytic activity. Thus, by comparison, WIN 1601 shows a five-fold increase in potency when converted to the methiodide salt whereas with WIN 1423 no significant change in activity was observed upon conversion to the quaternary (table 1). These compounds differ from each other only in the position of the methyl substituent on the side chain. Increase in anticholinergic activity with the conversion of a tertiary amine ester into its corresponding quaternary metho-salt has been described by Meier and Hoffmann (6) for diethylaminoethyl diphenylacetate and by Ing *et al.* (4) for diethylaminoethyl diphenylglycolate. The former was found to be 3.3 and the latter 25 times more active than the corresponding tertiary amine hydrochlorides. Similarly, Ing *et al.* (4) have compared the anticholinergic activity of atropine, 1-hyoscyamine, 1-hyoscyne and eucatropine with their metho-salts and have reported activity of 0.97 (1-hyoscyne methiodide) to 7.0 (eucatropine methiodide) times that of the corresponding tertiary amine. The order of increase in the anticholinergic activity of the above aminopropane derivative (WIN 118) upon conversion to its metho-salt more nearly resembles that of the above anticholinergic esters. The nitriles are

outstanding in showing a significantly greater increase in activity upon conversion to the quaternary methiodide salts.

Lehmann and Knoefel (7) have reported transient spasmodic in the dog following the intravenous administration of 0.05 mgm./kgm. of diethylaminoethyl fluorene-9-carboxylate hydrochloride. Necheles *et al.* (8) report slight spasmodic of the dog jejunum and colon after the intravenous administration of 0.33-0.36 mgm./kgm. of diethylaminoethyl diphenylacetate. Graham and Lazarus (9) reported the latter compound to be spasmolytic to the rabbit small intestine at an intravenous dose of 0.1 mgm./kgm. Dimethylaminoethyl di-n-butylcarbamate methosulfate in subcutaneous doses of 0.25 mgm./kgm. was found to be spasmolytic to the stomach and small intestine of unanesthetized dogs by Featherstone and White (10). The most active anticholinergic nitriles described here appear to be more effective than the aforesaid drugs inasmuch as intravenous doses as small as 0.01 mgm./kgm. caused spasmodic of the dog small intestine and colon. Similarly, the small intestine and colon of the anesthetized rabbit are effectively relaxed by intravenous doses of 0.01-0.05 mgm./kgm.

Action on other parasympathetically innervated structures suggests that these potent anticholinergic nitriles will have atropine-like side effects. However, because of the difference in sensitivity between man and experimental animals, it would be difficult to estimate their probable clinical toxicity prior to actual trial. Compared with other experimental data, it would seem that the inhibitory effect on salivary secretion is less than that reported by Ing *et al.* (4) for diethylaminoethyl diphenylglycolate but more than that for diethylaminoethyl diphenylacetate of fluorene-9-carboxylate (Lehmann and Knoefel, 7). Two compounds (WIN 1075 and WIN 1234) were approximately equal to atropine in their effect on salivary secretion.

Atropine sulfate appears to be at least ten times more active than WIN 1075 and WIN 1274 in blocking the vasodepressor actions of acetylcholine. These drugs were found to be approximately one-tenth as effective as atropine and two times as effective as homatropine in inducing mydriasis after topical application to the cat's eye.

Scopolamine-like intoxication was not observed with either WIN 1075 or WIN 1234, except at high doses. Abreu *et al.* (11) have reported marked intoxication in dogs following subcutaneous doses of 1.0 mgm./kgm. of diethylaminoethyl phenyl-2-thienylglycolate. The spasmolytic dose was reported to be 0.1 mgm./kgm.; thus the margin of safety found with this antispasmodic is much smaller than that found for the nitriles described in this communication. These nitriles more nearly resemble dimethylaminoethyl diphenylglycolate ethochloride which according to Ing *et al.* (4) produce symptoms of mild intoxication in cats when administered subcutaneously in doses of 30-60 mgm./kgm. These observations are in agreement with those reported by Fromherz (12) who found that a reduction in the central stimulating action of various anticholinergic esters occurs when the tertiary amine is converted to the quaternary methosulfate.

The methiodide salts of the nitriles were found to be distinctly more toxic than their corresponding hydrochlorides. In this respect, they resemble the

esters described by Meier and Hoffmann (6) wherein an increase in toxicity paralleled an increase in neurotropic (anticholinergic) potency resulting from the conversion to the quaternary salt. The increase in toxicity upon quaternizing these nitriles does not appear to be accompanied by a resultant enhancement of their atropine-like actions. Thus, WIN 1359 with a relative potency of 2 is more toxic than WIN 1263 with a relative potency of 24. Oral toxicity was determined for two of the compounds (WIN 1075 and WIN 1234) having high spasmolytic potency. Both were found to be much less toxic orally than intravenously. The effective oral dose of WIN 1075 was about 100 times the effective intravenous dose which suggests that the low oral toxicity results from slow absorption or rapid detoxification.

#### SUMMARY

1. The methiodide salts of various diphenylaminoalkanenitriles have significant anticholinergic activity.
2. The nitrile group appears to be important for this anticholinergic action.
3. The most active quaternary salts in this group of compounds are effective antispasmodics when administered parenterally, sublingually or orally.
4. Acute intravenous toxicity of these quaternary salts in albino mice is relatively high; by contrast oral toxicity of two of these is low.

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#### REFERENCES

1. BECKER, T. J., ANANENKO, E., GLENWOOD, G., AND MILLER, L. C.: *Fed. Proc.*, 5: Part II: 163, 1946.
2. MILLER, L. C., BECKER, T. J., AND TAINTER, M. L.: *THIS JOURNAL*, 92: 260, 1948.
3. JACKSON, D. E.: *Experimental Pharmacology and Materia Medica*, The C. V. Mosby Company, St. Louis, Mo., 2nd Edn., page 90, 1939.
4. ING, H. R., DAWES, G. S., AND WAJDA, I.: *THIS JOURNAL*, 85: 85, 1945.
5. SCHULTE, J. W., TAINTER, M. L., AND DILLE, J. M.: *Proc. Soc. Exper. Biol. and Med.*, 42: 242, 1939.
6. MEIER, VON R., AND HOFFMANN, K.: *Helv. Med. Acta*, 7: 106, Suppl. 6, 1941.
7. LEHMANN, G., AND KNOEFEL, P. K.: *THIS JOURNAL*, 74: 274, 1942.
8. NECHELES, H., OLSON, W. H., NEUWELT, F., AND SPIER, E.: *Gastroenterology*, 2: 46, 1944.
9. GRAHAM, J. D. P., AND LAZARUS, S.: *THIS JOURNAL*, 69: 331, 1940.
10. FEATHERSTONE, R. M., AND WHITE, N. G.: *THIS JOURNAL*, 84: 105, 1945.
11. ABREV, B. E., AND TROESCHER-ELAM, E.: *THIS JOURNAL*, 86: 205, 1946.
12. FROMHERZ, K.: *Arch. f. exper. Path. u. Pharmacol.*, 173: 86, 1933.
13. MILLER, L. C., AND TAINTER, M. L.: *Proc. Soc. Exper. Biol. and Med.*, 57: 261, 1944.

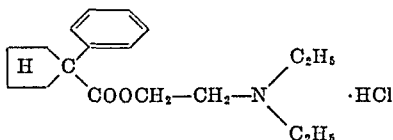
# THE EFFECT OF DIETHYLAMINOETHYL ESTER OF 1-PHENYL-CYCLOPENTANE-1-CARBOXYLIC ACID HYDROCHLORIDE, "PARPANIT", ON DECEREBRATE RIGIDITY, SPINAL REFLEXES AND SKELETAL MUSCLE<sup>1</sup>

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Diethylaminoethyl ester of phenylcyclopentane carboxylic acid hydrochloride, "Parpanit", has been introduced into clinical



medicine for the relief of symptoms caused by basal ganglion diseases (1). In these conditions, one of the more prominent and disturbing symptoms is muscle rigidity and upon this the drug has a pronounced effect. In guinea pigs the muscular rigidity of tetanus (2) can be completely suppressed by the use of Parpanit and in the dog, it depresses strychnine convulsions (3). Hartman (4) reports a marked decrease in muscle tonus following the administration of Parpanit in human beings.

In experimental animals Heymans and De Vlceschhouwer (3) reported a decrease in the muscular tonus especially of the abdominal muscles following the intravenous injection of Parpanit. While comparing the intensity of the visible contractions of the muscles of the paw before and after the injection of the drug either intravenously or intra-arterially, they concluded that such injections had no effect upon either the neuromuscular conduction at the level of the voluntary muscles, or on direct muscular excitability. Domenjoz (5), however, using a perfused gastrocnemius muscle of the frog, noted a decrease in general tonus, in the height of muscular contraction with small doses and a complete failure of the muscle to respond to nerve stimulation with larger doses. This latter effect he believes, without implying that the mechanism is the same, is suggestive of the effects of curare and atropine. Fleisch and Baud (6) studied the effect of Parpanit on muscle tonus and conclude that the drug acts on the peripheral proprioceptive elements of the muscles and joints which are responsible for maintaining muscular tonus.

Recently Lehner and Lanini (7) studied the effect of Parpanit on the metabolism of skeletal muscle cells. Slices either of the gluteus or of the quadriceps

<sup>1</sup> This work was made possible through a grant by the Geigy Company, Inc. for research in the sciences.

muscles of rats were used. In these they noted low concentrations to activate cellular respiration, moderate concentrations to have no effect and high concentrations to inhibit cellular metabolism.

Because of the above discrepancies in the literature, we thought a thorough investigation of the subject should be carried out and the following problems investigated: a) the effect of Parpanit on the rigidity of decerebrated animals; b) the effect on the reflexes of spinal animals; c) the effect on the contractions of a skeletal muscle of a warm blooded animal stimulated either through its nerve or directly, and; d) where indicated, a comparison of the effects of this drug with those of similar compounds, Syntropan and Trasentine and with atropine, scopolamine, d-tubocurarine and Intocostrin.

*Decerebrated Cats.* Sherrington's method of decerebration was employed. First the cats were anesthetized with ether, the carotid arteries ligated and a cannula placed in the trachea for artificial respiration, if needed, during decerebration. From this point the method was similar to that described by Forbes (8). The vertebral arteries were clamped off by applying pressure with the forefinger and thumb, one on either side of the neck, just below the transverse processes of the axis during the time that the cerebral hemispheres were being removed from the cranium, and until the blood in the wound was sufficiently coagulated to prevent hemorrhage. In the few cases in which the lack of blood to the respiratory center stopped normal respiration, artificial respiration was employed until the release of pressure on the vertebral arteries allowed normal respiration to return. After the bleeding had ceased, the pressure on the vertebral arteries was gradually released, the skull loosely filled with absorbent cotton, the skin flap replaced and the cut skin surfaces clipped together securely. Seventeen animals were used.

The animal was placed on its back on the animal board with the extremities unimpeded. Eight to forty-five minutes were allowed to pass for the rigidity of the muscles to become maximum, before any intravenous injections were made.

Following each of the injections of Parpanit the rigidity of the decerebrated cats was either reduced or abolished. A visible effect occurred within one minute after the injection and a maximum response was seen three to ten minutes later. In all instances, the initial injection appeared to have a greater effect than subsequent injections. Two mgm./kgm. of Parpanit were injected five times and in these nearly complete relaxation occurred in two instances and in the remainder the abolition of the rigidity appeared to be complete. Three mgm./kgm. were given intravenously five times and in all but one case practically complete disappearance of the rigidity was observed. Five mgm./kgm. of Parpanit were given intravenously fifteen times and 10 mgm./kgm. once. Following all of these injections the muscles appeared to be completely relaxed (see figure 1). In three animals in which spontaneous muscle tremors of the hind limbs were present these were no longer observed after the injections. Atropine sulfate in 0.5 and 5 mgm./kgm. doses and scopolamine hydrobromide in 1 and 2 mgm./kgm. doses produced either no change in the rigidity of the muscles or only a slight relaxation. Parpanit, Trasentine and Syntropan administered in 5 mgm./kgm. doses, subsequently, caused marked to complete disappearance of the muscular rigidity proving these substances to be more powerful than either atropine or scopolamine in relieving this condition.

Trasentine in 5 and 10 mgm./kgm. doses was given intravenously five and one times, respectively, and Syntropan was similarly administered four times in doses of 5 mgm./kgm. In all instances, practically complete disappearance of the muscular rigidity occurred soon after the injection.

*Reflexes in Spinal Animal.* The surgical procedure here was similar to that used in decerebration except that the head was completely removed after ligating the carotid arteries, external and internal jugular veins and compressing the vertebral arteries by means of a wire securely fastened about the neck. Artificial respiration was used to maintain the life of the decapitated animal. Within 30 to 40 minutes the reflex responses to mechanical stimulation of the skin and paws were usually excellent.

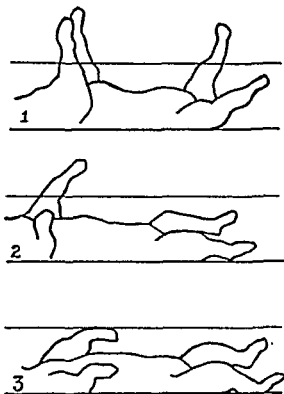


FIG. 1. Cat ♂ 2.8 kgm. decerebrated under ether anesthesia. A line drawing, side view. 1. Control. The limbs are rigid and extended due to muscular rigidity. 2. The limbs are falling by their own weight after the injection of 5 mgm./kgm. Parpanit intravenously. 3. Three minutes later. The extremities are flaccid, the muscles relaxed and the limbs are now resting against the body and on the animal board. The rigidity of the muscles as seen in 1, had returned 29 minutes later.

Only four cats were used in this study. Five mgm./kgm. of Parpanit were injected intravenously five times and in these there appeared to be some weakening of the reflexes especially the scratch reflex although the results were not too convincing. Ten mgm./kgm. of Parpanit were given three times and in these the reflexes were markedly depressed in each case. Trasentine and Syntropan were injected intravenously in 10 mgm./kgm. doses once each in one animal. The reflex response to mechanical stimulation of the skin disappeared immediately as it did following Parpanit administration.

*Nerve Muscle.* Four cats, decerebrated as outlined above, one cat under urethane anesthesia, and twenty-one dogs anesthetized with urethane (1.5 gm./kgm. intraperitoneally) were used in these experiments. By making a small slit through the skin on the outer side of either thigh, the *peroneus communis* nerve was bared and along its entire length separated



from the tibialis nerve.<sup>2</sup> The nerve was cut, and its distal end fastened in a Sherrington shielded electrode (9). The electrode was then held in place by fastening around it, with metal clips, the two flaps of skin. Through another slit in the skin the tendon of the *tibialis anticus* muscle corresponding to the nerve to be stimulated was isolated from its insertion. The tendon was then fastened to a muscle lever by a string passing about a series of pulleys. The pulleys were arranged so that the muscle pulled in its normal direction. One thong looped about the hock and another around the foot just below the fastening of the tendon bound the leg securely to the animal board and made a very satisfactory muscle preparation (10). This preparation had its normal blood supply unaltered except for the cutting of the *peroneus communis* nerve.

In those experiments in which the muscles were stimulated directly the skin and underlying tissues were cut away from the *tibialis anticus* muscle in two places about 3 cm. apart. Through these small openings platinum needle electrodes were thrust into the muscle.

One sciatic nerve was cut in each of five dogs and one cat under ether anesthesia five to ten days before the experiment was performed to permit degeneration of the nerve to the muscle. In four additional dogs 10 units of Intocostarin were injected intra-arterially, the dose being repeated as needed to paralyze the motor nerves to the *tibialis anticus* muscles. In these experiments the muscle was stimulated directly as outlined above.

The stimulating currents were either maximal break induction shocks obtained from a "Harvard" inductorium or a current obtained from an "Electrodyne stimulator". With the latter apparatus a current of 1 to 4 volts was used for the nerve and the rate of interruption was either 60 or 120 per minute the number being constant for each experiment. When a tetanizing current was employed its rate of interruption was 30 per second. With this current the nerve was stimulated for two seconds every thirty seconds. For direct excitation of the muscle either a current of 250 volts from the "Electrodyne stimulator" or maximal induction shocks from the inductorium were used with a rate of interruption of 60 times per minute. The muscle lever consisted of a piece of light straw with a celluloid tip, 20 cm. in length from the axis to the writing point. The tendon was attached 2 cm. from the axis and at the moment the contraction began it pulled against the tension of a spring, which was 50 gm. and for each centimeter excursion of the lever on the drum the spring tension increased 5 gm.

The femoral artery, on the same side as the muscle being used, was exposed for intra-arterial injection. In some experiments injections were also made into the external jugular vein. When a constant slow rate of injection was indicated an injection machine operated by a synchronous motor was employed. In all cases except those of Intocostarin and d-tubocurarine, the drugs were dissolved in 0.9 per cent sodium chloride solution the concentration being 10 mgm. of the drug in each cc. of the solution.

**RESULTS.** Due to the fact that Parpanit in large doses given intravenously lowers the blood pressure and this in itself would tend to decrease the height of muscular contraction (11), only a few experiments were performed using this route of administration.

In cats, 3 mgm./kgm. were given intravenously once and 5 mgm./kgm. twice. Following each injection a small increase in the height of the muscular contractions occurred. In dogs only two intravenous injections of Parpanit (10 mgm./kgm.) were made and in these a definite increase in the force of the contractions of the *tibialis anticus* muscle was noted.

Best results were obtained with intra-arterial injections of the drugs. Twelve such injections of Parpanit (1, 1 mgm.; 3, 3 mgm.; 2, 5 mgm.; 5, 10 mgm.; 1, 20 mgm.) were made in four cats and sixty-six similar injections (1, 0.5 mgm.;

<sup>2</sup> The nomenclature employed is that of Reighard and Jennings, *The Anatomy of the Cat*, New York, 1901.

1, 0.6 mgm.; 2, 1 mgm.; 6, 2 mgm.; 14, 3 mgm.; 10, 5 mgm.; 30, 10 mgm.; 1, 15 mgm.; 2, 20 mgm.; 2, 50 mgm.) were made in 17 dogs. No differences were noted in the results obtained with the inductorium and with the "Electrodyné

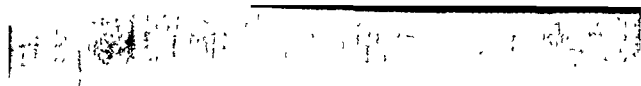


FIG. 2 Dog ♂ 8 kgm. under urethane anesthesia. Top record is that of the *tibialis anticus* muscle contracting 120 times a minute, stimulated through its nerve. Bottom record the blood flow from deep tibial vein in 10 cc. volumes. Middle record the time in intervals of 20 seconds. At 2, in the record, 3 mgm. of Parpanit were injected into the femoral artery supplying the muscle

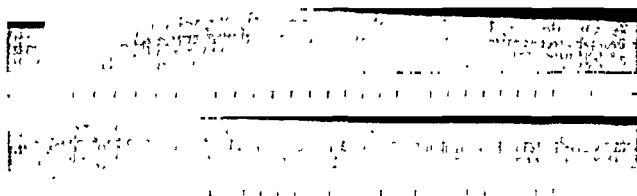


FIG. 3. Dog ♀ 10 kgm. under urethane anesthesia. The top record in each curve is that of the *tibialis anticus* muscle excited through its nerve 120 times a minute. The current was 4 volts. Below each muscle's record is the time in intervals of 20 seconds. At 6, 10 mgm. of Parpanit were injected slowly into the femoral artery supplying the muscle. At 7, the same dose was injected rapidly.



FIG. 4. Cat under ether anesthesia. Top record is that of the *tibialis anticus* muscle contracting 60 times a minute. Stimulating current was 1 volt. Below each muscle's record is the time in 20 seconds. At 2, in the record, 10 mgm. of Parpanit were injected slowly into the femoral artery supplying the muscle.

stimulator" and between single shocks and tetanizing currents. Large amounts of Parpanit injected slowly (figures 2 and 3) gave the same results as small doses (0.5 to 3 mgm.) (figure 4) injected rapidly. Moreover, the size of the animal, i.e., the muscular development supplied by the deep tibial artery, also appeared

from the tibialis nerve.<sup>2</sup> The nerve was cut, and its distal end fastened in a Sherrington shielded electrode (9). The electrode was then held in place by fastening around it, with metal clips, the two flaps of skin. Through another slit in the skin the tendon of the *tibialis anticus* muscle corresponding to the nerve to be stimulated was isolated from its insertion. The tendon was then fastened to a muscle lever by a string passing about a series of pulleys. The pulleys were arranged so that the muscle pulled in its normal direction. One thong looped about the hock and another around the foot just below the fastening of the tendon bound the leg securely to the animal board and made a very satisfactory muscle preparation (10). This preparation had its normal blood supply unaltered except for the cutting of the *peroneus communis* nerve.

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<sup>2</sup> The nomenclature employed is that of Reighard and Jennings, *The Anatomy of the Cat*, New York, 1901.

seconds and after recovery from this depression the contractions are no higher than they were before the injection of the drug.

Intocostrin and d-tubocurarine were administered in the artery supplying the muscle 28 times (one each 0.05, 0.02, 0.1, 0.2 units; 3, 0.3; 3, 0.5; 12, 1; 3, 3; 1, 5; 2, 10 units) in 12 dogs. In these a decrease in the height of muscular contractions was noted only in those muscles in which the nerves were initially active. In all instances the recovery of the muscle from curare was very slow, the height of the muscular contractions rarely returning to their original value and never exceeding the control level. No changes in the height of muscular contraction were noted after the intra-arterial injections of either Intocostrin or d-tubocurarine in denervated muscles (muscles to which the nerves had been cut 5 to 10 days) a response which is quite different from that obtained following similar injections of Parpanit, Trasentine and Syntropan.

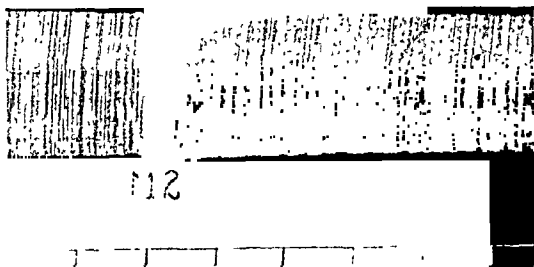


FIG. 5. Dog ♀ 10 kgm. under urethane anesthesia. Top record is that of the anterior tibial muscle contracting 120 times a minute and below it the time in intervals of 20 seconds. At 12, 10 mgm. of atropine sulfate were injected intra-arterially.

*Denervated Muscles.* To determine if Parpanit and Trasentine depress the neuro-muscular mechanism as do Intocostrin and d-tubocurarine or if they act directly on the muscle substance experiments were performed on muscles to which the nerves had been cut 5 to 10 days previously and also on some muscles in which the nerves were paralyzed by intra-arterial injections of Intocostrin. None of these muscles responded to nerve excitation.

Atrophy by disuse of the muscle appears to have a decided effect upon the excitability of the muscle and upon its responsiveness to Parpanit and Trasentine. In those animals in which the nerve to the muscle had been cut 9 to 10 days either strong currents (250 volts) or strong induction shocks were necessary to excite the muscle and the contractions were extremely weak. Although dilatation of the blood vessels in the muscle occurred, nevertheless increases in the height of the contractions of the muscle were difficult to obtain with the use of Parpanit. Depression of the muscle was usually seen. In those animals in which the nerve had been cut 5 days, doses of Parpanit which would ordinarily depress normal muscle still caused an increase in the height of the contractions

(see figure 6). In this experiment 80 mgm. of Parpanit were injected intra-arterially rapidly at 7. The usual increase in the height of the contractions occurred but a slight temporary depression may also be seen. In this animal which was quite large (14.5 kgm.) 1 mgm. of Parpanit similarly injected had no effect, however, 3, 10, 20 and two 40 mgm. injections caused only increases in the muscular activity. The results as a whole with the denervated muscles were similar to those obtained with normal muscles. That these muscles were probably completely denervated was shown by the fact that the intra-arterial injections of Intocostin in doses of 1 to 10 units had no effect upon the activity of the muscle, although Intocostin, like Parpanit, caused an increase in blood flow from the femoral vein.

Parpanit was injected intra-arterially ten times (2, 10 mgm.; 8, 20 mgm.) and Trasentine once (20 mgm.) in four dogs following intra-arterial injections of Intocostin. In these an increase in the height of the contractions of the muscle was seen in four cases; a temporary decrease followed by an increase in two; and a decrease in three. The lone injection of Trasentine produced a temporary decrease in muscular activity, followed by a prolonged increase in the height of the contractions. These results confirm our results on denervated muscles (nerves degenerated) and those in which the nerve was active and stimulated



FIG. 6. Dog ♂ 14.5 kgm. under urethane anesthesia. The sciatic nerve to the muscle was cut 5 days before this experiment was done. The muscle failed to respond to strong nerve stimulation. Top record is that of the contractions of the denervated muscle stimulated directly (250 volts) 60 times a minute and below it the time in intervals of 20 seconds. At 7, 80 mgm. of Parpanit were injected intra-arterially.

**Blood Flow.** Since Domenjoz (5) observed dilatation of the vessels in the rabbit's ear after the administration of Parpanit, and Heymans and De Vleeschhouwer (3) noted peripheral vasodilatation of the extremity by the two manometer method we believed it important to determine whether Parpanit causes dilatation of the blood vessels in the skeletal muscle from which we were recording, and if so, whether this could account for the marked improvement in the activity of the muscle following intravenous and intra-arterial administrations.

In these experiments the changes in blood pressure were registered from the right carotid artery by means of a mercury manometer using heparin in the system as the anticoagulant. A glass cannula was placed in the femoral vein, all the branches to which were tied off except the deep anterior tibial vein which comes from the *tibialis anticus* muscle and the other muscles of that region. The cutaneous vessels to the limb were fairly well controlled by the thongs drawn tightly around the foot for fixing the leg. In order to maintain a fairly constant blood pressure level in these experiments the blood as it left the muscle was re-injected into the animal through a cannula placed in the right external jugular vein. Coagulation of the blood was prevented by giving the animal heparin 15 mgm./kgm. intravenously and repeating as needed. The blood flowing from the muscles was measured in 10 cc. volumes and these units were recorded on the drum surface with a signal magnet.

Twenty-three injections of Parpanit (1, 0.6 mgm.; 10, 3 mgm.; 4, 5 mgm.; 8, 10 mgm.) were made in five dogs. The results were so uniform that work on additional animals was thought unnecessary. With the exception of the smallest dose, in every instance the volume of the blood leaving the femoral vein was approximately double the flow before the injection. The results were the same whether the activity of the muscle was increased or decreased by the drug. Figure 2 is such a record. At 2, 3 mgm. of Parpanit were injected into the femoral artery supplying the muscle. The contractions of the muscle increased 33 per cent in height but the volume of blood leaving the femoral vein increased 120 per cent, i.e., the volume increased from 20 cc. per minute to 50 cc. per minute immediately after the injection.

Three intra-arterial injections of Trasentine were made and the results were the same as those produced by Parpanit.

**Discussion.** Our results suggest that the depression of decerebrate rigidity and of spinal reflexes by Parpanit, Trasentine and Syntropan is a central effect. The concentrations needed to reduce the contractions of skeletal muscle are far greater than those needed to depress the reflexes and relieve decerebrate rigidity. Moreover, doses which produce these effects are either stimulating or without action on the artificially stimulated muscle.

The increase in the height of muscular contraction following the injection of Parpanit, Trasentine and Syntropan is probably independent of the increase in blood flow. Gruber (10) demonstrated a two- to three-fold increase in the blood flow through the anterior tibial muscle of cats following the cutting of its nerve. Moreover, in these experiments since the muscle was made to work by stimulating its nerve rhythmically thus increasing its blood supply it appears to us that the rate of blood flow through the muscle was already adequate to supply the necessary oxygen and food and remove all metabolites before the injection of the drug. In these animals, atropine and sodium nitrite were injected intra-arterially producing marked increases in the blood flow through the muscle without causing a change in the height of the muscular contractions. Furthermore, small and large doses of Intocostrin caused marked dilatation followed by constriction of the blood vessels in skeletal muscle yet no increase in the muscular activity was ever observed. In the light of studies by Lehner and Lanini (7) we believe the increase in the height of the muscular contractions to be a direct effect of these drugs on skeletal muscle cells, i.e., an increased cellular metabolism occurring with the increased muscular activity and a decrease in the cellular metabolism with the decrease in the height of the contractions. That this action is not on the neuro-muscular junction is demonstrated by the fact that both increased and decreased height of muscular contractions occur in denervated muscles following the intra-arterial injection of Parpanit.

#### CONCLUSIONS

1. Parpanit, Trasentine and Syntropan, injected intravenously in adequate doses, relieve the muscular rigidity of decerebrated cats. For this purpose these

drugs were found to be superior to either atropine sulfate or scopolamine hydrobromide.

2. The reflexes caused by stimulation of the back and paws in the spinal cat can be depressed by adequate doses of Parpanit, Trasentine and Syntropan given intravenously.

3. The height of the contractions of the anterior tibial muscle is increased with the intravenous injections of Parpanit.

4. The intra-arterial injections of Parpanit, Trasentine and Syntropan increase the height of the contractions of the anterior tibial muscle when their doses are small and rapidly injected and when their doses are large and slowly injected. Large doses rapidly injected cause a decrease in the activity of the muscle preceded and followed by increases above the height of the control. Atropine sulfate, Intocostin and d-tubocurarine when injected intra-arterially decrease muscular activity but this decrease is never followed by an increased action of the muscle.

5. Parpanit, Trasentine, Syntropan, atropine sulfate, and Intocostin cause vaso-dilatation of the blood vessels in skeletal muscle when injected intra-arterially.

6. The increased and decreased muscular activity following injections of Parpanit and Trasentine are probably direct effects of these drugs on the muscle cells, independent of the increase in blood flow, increasing cellular metabolism in some instances (small doses) and temporarily decreasing cellular metabolism in others (large doses).

7. The depressant effect of Parpanit is not on the neuromuscular junction as is that of Intocostin and d-tubocurarine.

#### REFERENCES

1. DOMENJOZ, K.: Schw. Med. Wschr., 76: 1282, 1946; HARTMAN, K.: Schw. Med. Wschr., 76: 1289, 1946 and Therap. Umschau, 3: 163, 1947; GRUNTHAL, E.: Schw. Med. Wschr., 76: 1986, 1946; BICKEL, G., AND DUBOIS, J. P.: Rev. Med. de la Suisse Romande, 67: 708, 1947; SCHWAB, R. S., LEIGH, D., AND COBB, C. T.: Paper presented at the 73rd Meeting of the Am. Neurol. Assn., June 16, 1948.
2. HARTMANN, K.: Therap. Umschau, 3: 163, 1947.
3. HEYMANS, C., AND DE VLEESCHOUWER, G. R.: Arch. Internat. de Pharmacodyn. et de Therap., 76: 307, 1948.
4. HARTMANN, K.: Schw. Med. Wschr., 76: 1289, 1946.
5. DOMENJOZ, K.: Schw. Med. Wschr., 76: 1282, 1946.
6. FLEISCH, A., AND BAUD, C.: Helv. Physiol. et Pharmacol. Acta, 6: 331, 1948.
7. LEHNER, F., AND LANINI, G.: Helv. Physiol. et Pharmacol. Acta, 6: 346, 1948.
8. FORBES, A.: Quart. J. Exper. Physiol., 5: 166, 1912.
9. SHERRINGTON, C. S.: J. Physiol., 38: 382, 1909.
10. GRUBER, C. M.: Am. J. Physiol., 45: 306, 1918.
11. GRUBER, C. M.: Am. J. Physiol., 32: 221, 1913.

# OBSERVATIONS ON THE DEVELOPMENT OF TOLERANCE DURING PROLONGED ORAL ADMINISTRATION OF DIHYDROERGOCORNINE<sup>1</sup>

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Recent investigations have demonstrated that following the acute administration of two dihydrogenated alkaloids of ergot, dihydroergocornine<sup>3</sup> (DHO) and dihydroergokryptine<sup>1</sup> (DHK), there may be a significant fall in blood pressure and slowing of the pulse rate in certain hypertensive patients (1, 2). The reduction in arterial pressure is accompanied by postural hypotension, as well as by inhibition of certain sympathetic vasoconstrictor reflexes. Other drugs, such as dibenamine (3), and tetraethylammonium ion (4, 5), as well as certain procedures, including spinal (6) or caudal anesthesia (7) also have been shown to reduce blood pressure and inhibit sympathetic vasopressor responses. However, since such agents must be given by injection they are not practical for prolonged administration. Because in acute experiments the dihydrogenated alkaloids of ergot were found to be effective orally in sub-toxic doses (1, 2, 6), they appeared practicable for long continued clinical trial in hypertensive patients.

Preliminary experience with DHO indicated that the hypotensive action of the drug was seldom as marked after chronic oral administration as after acute intravenous or oral administration. The arterial pressure returned to, or toward the original levels presumably because of one, or both, of two effects: (1) the development of tolerance to the drug; or (2) the activation of other mechanisms, humoral or neurogenic, capable of restoring the arterial pressure to higher levels. That tolerance to ergot alkaloids may develop in animals has been suggested by others (8-11). The present study therefore, was undertaken to determine the nature of the tolerance that apparently develops after continued oral administration of the drug.

**METHODS.** In order to determine the development of tolerance the general plan of study was to compare the hemodynamic effects of intravenously administered DHO both before and after the chronic oral administration of the drug. It was reasoned that if tolerance developed during chronic administration the second intravenous dose of DHO would fail to reproduce the hemodynamic effects of the initial dose. Particular emphasis was placed

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<sup>3</sup> Hereinafter dihydroergocornine will be referred to as DHO and dihydroergokryptine as DHK.



upon the changes observed in the arterial pressure, heart rate, and sympathetic vasomotor reflexes.

Five patients with essential hypertension who could be studied at frequent intervals as out-patients were selected and given oral DHO in doses of 4 to 12 mgm. a day for periods ranging from 8 to 114 days. After establishing the pattern of an untreated patient's response to his first intravenous dose of DHO, he was placed on continued oral medication with the drug. Following 3 to 15 weeks of such treatment he was given both DHO and DHK in separate intravenous doses and the results compared to the intravenous response prior to oral administration. Throughout the periods of observation 4 patients had no associated treatment, while one, J. Mc., received *Veratrum viride* and *digitalis* in conjunction with the oral DHO.

To establish the fact that tolerance would not develop after single repeated intravenous doses of DHO, 2 patients were given identical injections of DHO intravenously on 2 separate occasions at intervals of 4 days and 4 weeks, respectively. In both cases there were essentially similar hypotensive responses on the 2 occasions. Furthermore, in 3 cases oral DHO was withdrawn for 3 to 4 weeks after tolerance had been established and intravenous DHO administered at that time again resulted in hypotensive and hemodynamic responses similar to those noted on the initial test dose before oral medication.

Unless otherwise stated the arterial pressure was recorded 5 or more times with a cuff and mercury manometer, after the patient had been resting in bed comfortably for a minimum of 15 minutes. Arterial pressure in the erect position was measured in the same manner after the patient had been standing for 1 minute. Repeated observations of supine and erect arterial pressure and heart rate were made at 3- to 10-minute intervals for a minimum of 60 minutes following an intravenous injection of a drug. In 2 cases arterial pressure and heart rate were recorded with a Hamilton manometer (12).

Assessment of sympathetic vasopressor activity was evaluated in three ways: 1. By the *Valsalva maneuver* as described by Wilkins and Culbertson (13). In 2 cases, the response was measured with a Hamilton manometer. In the 3 remaining cases the Valsalva response was estimated with a pneumatic arm cuff. The *systolic overshoot* was determined by instructing the patient to blow against a fixed pressure several times. Immediately upon cessation of forced expiration, the pressure in the cuff was raised by increments of 10 mm. Hg above the basal systolic levels until the peak of the overshoot was determined. Since the vasopressor overshoot following the Valsalva maneuver may be blocked either by surgical sympathectomy (13), or by sympatholytic agents (14), a measure of the degree of sympathetic blockade may be calculated and expressed in per cent for comparative purposes.

2. By the *degree of postural hypotension* (15). To express the degree of postural hypotension, the mean arterial blood pressure (one-half the sum of the systolic plus the diastolic) was calculated for both the supine and upright positions. The difference of the mean values, in per cent, was determined as an expression of the blood pressure response to orthostasis. The values were again determined at the height of the hypotensive reaction to the intravenous administration of the DH-alkaloid. The algebraic sum of the pre- and post-drug percentile responses to orthostasis was used as a measure of the sympatholytic action of the drug.

3. By the *cold pressor response*. Although this response may be a less reliable measurement of sympathetic nervous reactivity, it has been shown usually to be inhibited by the DH-alkaloids (2), as well as by other sympatholytic agents (3, 16). The test as described by Hines and Brown (17), was modified in that the highest blood pressure reading obtained during immersion of the hand in ice water for 1 minute was designated as the cold pressor response.

**RESULTS.** *The Development of Tolerance to DHO.* 1. *Blood Pressure and Heart Rate.* Before beginning chronic oral medication the 5 patients had reductions in arterial pressure after their initial doses of 0.25 mgm. of DHO intra-

venously which varied from  $-8$  to  $-12$  per cent in 4 patients and  $-25$  per cent in 1. The mean pulse rate also decreased, averaging  $-23$  per cent in 3 cases and  $-10$  per cent in 2 cases (figure 1)

In contrast after a period of continued oral medication with DHO the same dose of drug administered intravenously exhibited little effect upon arterial pressure. Only negligible changes of  $+2$  to  $-3$  per cent occurred thereby indicating that tolerance to the drug had developed. However, despite this apparent loss of hypotensive action, DHO intravenously caused reductions of heart rate ranging from  $-8$  to  $-17$  per cent in all cases (figure 1)

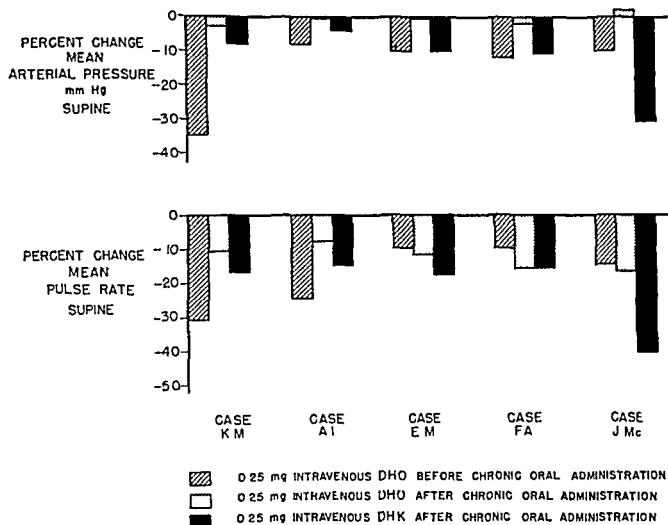


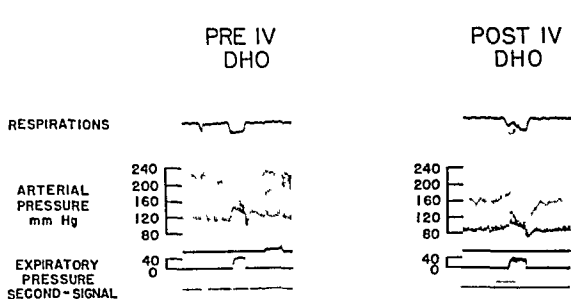
FIG 1 Graphic representation of the comparative reductions in blood pressure and pulse rate after intravenously administered DH-alkaloids in 5 hypertensive patients before and after chronic oral administration of DHO

**2. Sympathetic Vasoconstrictor Reflexes. a. The Valsalva Test.** Prior to oral administration of DHO, the vasopressor overshoots following the Valsalva maneuver which in control tests ranged from 10 to 28 per cent were reduced by intravenous injection of 0.25 mgm. of the drug to complete abolition in 3 of 4 cases tested and from 21 to 7 per cent in 1 case. In contrast, the overshoot response returned almost completely in 3 cases, partially in 1 and remained blocked in 1 case during continued oral administration. At that time the same dose of DHO intravenously produced less inhibition of the overshoots than it had initially ranging from 7 to 12 per cent in 4 cases (table I, figure 2). In the



remaining case, J Mc, whose treatment included *Veratrum viride* plus digitoxin, the overshoot remained blocked and as a consequence there could be no further inhibition after intravenous DHO at that time. It should be noted, however,

Before chronic oral Dihydroergocornine



After chronic oral Dihydroergocornine

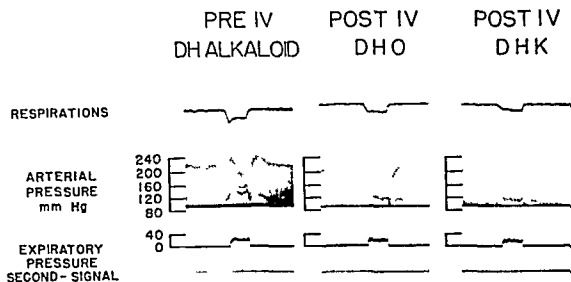


FIG. 2 Optical records of the vasopressor responses following the Valsalva maneuver in a hypertensive patient K M before and after the chronic oral administration of DHO. The upper records demonstrate the blockade of the overshoot after intravenous DHO prior to oral medication. Note the hypotension. The lower records demonstrate the return of the overshoot after chronic oral medication and its maintenance after intravenous DHO or DHK.

that 4 days later, prior to the administration of DHK, an overshoot had reappeared, despite continued oral administration of DHO.

b *Postural Hypotension* Intravenous injection of DHO prior to chronic oral medication was followed by significant postural hypotension in 4 of the 5 cases (table II). The orthostatic changes in mean arterial pressure were -6 to -9 per cent in 3 of these 4 cases, and -22 per cent in 1. In the remaining case there was

no significant postural effect. However, during the period of continued oral medication with DHO, the orthostatic hypotension disappeared and failed to reappear after the same intravenous dose which had induced significant postural changes prior to oral administration (table II). These results indicated that with the apparent loss of supine hypotensive effect of DHO during chronic oral administration there was also loss of the orthostatic action of the drug when given intravenously at that time.

c. *The Cold Pressor Test.* Prior to continued oral medication the intravenous injection of 0.25 mgm. of DHO reduced the vasopressor responses to the cold pressor test from an average of 19 per cent (range 12 to 29 per cent) to an average of 11 per cent (range 8 to 14 per cent) in 3 of the 4 cases tested and completely abolished the pressor response in the remaining case (table III). During chronic oral administration this response returned completely in 4 of the 5 cases. In case F. A., there was return of the pressor responses to cold which was noted visually

TABLE II

*Per cent change in postural hypotension following intravenous DH-alkaloids before and after the chronic oral administration of dihydroergocornine*

PATIENT	BEFORE CHRONIC ORAL ADMINISTRATION	AFTER CHRONIC ORAL ADMINISTRATION	
	DHO	DHO	DHK
	Per cent change postural hypotension	Per cent change postural hypotension	change
K. M. ....	-22.0†	+2.0†	†
A. I. ....	-7.0†	-0.8†	†
E. M. ....	-6.0†	-0.4†	
F. A. ....	-9.0†	+2.0†	
J. Mc. ....	+0.4†	+2.0†	

† Notations as in table I.

‡ Sphygmomanometric recordings of arteri

during the Hamilton recordings, but due on the film, and hence, could not be meas-

During chronic oral treatment, DHO significant inhibition of the cold pressor who had shown complete abolition after t was incomplete abolition, the response t the remaining case, the per cent inhibition tially (table III). Thus, intravenous D administration had produced a significant bl after chronic oral medication produced no in 3 cases, lessened inhibition in 1 case, and 1 case.

*The Effect of Intravenous DHK After Chronic* determine whether the tolerance resulting f.

TABLE III

The effect of intravenous *DH*-alkaloids on respressor overshoots following the cold pressor test before and after chronic oral administration of dihydroergocornine

PATIENT	BEFORE CHRONIC ORAL ADMINISTRATION						AFTER CHRONIC ORAL ADMINISTRATION					
	Pre-I.V. DHO			Post-I.V. DHO			Pre-I.V. DHO			Post-I.V. DHO		
	Arterial Pressure* mm. Hg		Per cent overshoot	Arterial Pressure* mm. Hg		Per cent overshoot	Arterial Pressure* mm. Hg		Per cent overshoot	Arterial Pressure* mm. Hg		Per cent overshoot
	Basal	Over-shoot		Basal	Over-shoot		Basal	Over-shoot		Basal	Over-shoot	
K. M.	218/140†	214/162	12.0	160/85	150/81	0	222/108†	218/124	11.0	212/104	220/110	4.0
A. L.	200/90†	270/118	29.0	183/70	214/108	14.0	201/102†	256/134	23.0	200/100	222/110	11.0
E. M.	182/92†	220/110	17.0	170/82	178/98	8.0	170/88†	210/110	17.0	178/98†	210/103	20.0
F. A.	210/142†	310/180	22.0	222/122	240/142	10.0	—‡	—	—	235/124†	274/124	22.0
J. Mc. S.	262/170†	310/200	15.0	—	—	—	210/118†	244/160	18.0	210/122	230/156	14.0

\*, †, § Notations as in table I.

‡ Technical failure of recording apparatus during control period.

† Sphygmomanometric recordings of arterial pressure.

other DH- alkaloids, intravenous DHK was administered in doses of 0.25 mgm. when tolerance to DHO apparently had developed. After the injection of DHK at that time there was significant lowering of the supine blood pressure in all cases ranging from  $-4$  to  $-31$  per cent (figure 1). As had been observed with DHO, slowing of the pulse rate occurred in all cases ranging from  $-15$  to  $-41$  per cent (figure 1).

Despite this reduction in arterial pressure after intravenous DHK, the pressor responses to the Valsalva maneuver and the cold test were not blocked (table I and III, figure 2), although there was significant orthostatic hypotension in all cases ranging from  $-8$  to  $-19$  per cent (table II).

DISCUSSION. These studies demonstrated that following continued oral administration of DHO a tolerance developed so that an intravenous injection of the alkaloid failed to produce as great a hypotensive effect as the same dose given prior to oral administration. Since, in addition, postural hypotension disappeared while the sympathetic vasopressor responses to such stimuli as the Valsalva maneuver and the cold pressor test returned, it seemed evident the tolerance to oral DHO included the sympatholytic as well as the hypotensive effects of the drug.

The moderate to marked reduction in supine arterial pressure and the reappearance of postural hypotension after intravenous injection of DHK was surprising in view of the fact that at this time the drug apparently did not produce sympatholytic effects as determined by the Valsalva maneuver and the cold pressor test; and suggested that vasodilatation occurred via some mechanism other than inhibition of sympathetic vasoconstriction. A possible explanation for this discrepancy may lie in the fact that the ergot alkaloids have varied sites of action, which include peripheral sympatholytic and adrenolytic actions as well as central points of attack (18, 19). Under such circumstances it is conceivable that tolerance to the sympatholytic effect extended to all the DH-alkaloids. However, tolerance at the other sites of action may not have included both DH- alkaloids, but indeed, may have been limited to each specific alkaloid. This would imply that the fall in supine pressure and postural hypotension after intravenous DHK was not due primarily to the sympatholytic effects of the drug but to some other action resulting from possible central, or peripheral, non-specific vasodilating properties, the exact nature of which is obscure.

Throughout the entire study persistent slowing of the pulse rate was observed whenever intravenous DHO, or DHK was injected, suggesting that chronic oral administration did not lead to the development of tolerance to this response. Since tolerance had developed to peripheral sympathetic vasopressor activity, the bradycardia occurring at this time seemed more likely due to a central effect upon the cardioinhibitor center, rather than to inhibition of the sympathetic cardioaccelerator nerve endings.

Bluntschli and Goetz concluded from their studies that small doses of DHO intravenously were depressor, but doses of 0.5 mgm. or higher were frequently pressor (1). Thus, it is conceivable that the apparent loss of hypotensive activity

following chronic oral administration could be explained as a cumulative effect leading to pressor action of the drug. However, even in relatively high oral doses, up to 12 mgm. a day, no *hypertensive* activity appeared. In addition, we have never observed that small doses have greater hypotensive effect than large doses, unless the latter produced disturbing side reactions such as nausea and vomiting. Finally, after chronic oral administration, intravenous doses of 0.25 mgm. DHO plus 0.25 mgm. DHK within one-half hour of each other produced no rise in blood pressure. These results mitigated against the idea that cumulation of the drug produced the observed loss of hypotensive activity.

#### SUMMARY AND CONCLUSIONS

When the same dose (0.25 mgm.) of DHO was administered intravenously before and after continued oral medication with the drug to 5 hypertensive patients, the following observations were made:

1. After continued oral medication with DHO, the intravenous injection of this drug no longer produced a significant hypotensive response.

2. Prior to continued oral administration significant inhibition of reflex sympathetic vasoconstriction, as determined by the responses following the Valsalva maneuver, cold pressor test, or the development of postural hypotension followed the intravenous administration of DHO. However, during continued oral administration of the drug the sympathetic vasoconstrictor reflexes returned and seldom were significantly inhibited by subsequent intravenous DHO. Thus, in addition to the loss of hypotensive activity, a partial to complete tolerance to the sympatholytic effects of DHO apparently had developed.

3. After the development of tolerance to DHO, the intravenous injection of DHK, a closely related compound, produced a reduction in arterial pressure, and postural hypotension, but failed to cause significant inhibition of the Valsalva or cold pressor responses.

4. During chronic oral medication with DHO the intravenous administration of either DHO or DHK resulted in a reduction in heart rate similar to that observed initially.

5. It is suggested that the DH- alkaloids have varied sites of action and that tolerance did not extend to all of the pharmacologic properties of these drugs.

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#### REFERENCES

1. BLUNTSCHLI, H. J., AND GOETZ, R. H.: *South African Med. J.*, **21**: 382, 1947.
2. FREIS, E. D., STANTON, J. R., AND WILKINS, R. W.: *Am. J. Med. Sci.*, **216**: 163, 1948.
3. HECHT, H. H., AND ANDERSON, R. B.: *Am. J. Med.*, **3**: 3, 1947.
4. ACHESON, G. H., AND PEREIRA, S. A.: *THIS JOURNAL*, **87**: 273, 1946.
5. MOE, G. K.: *J. A. M. A.*, **137**: 115, 1948.
6. PAGE, I. H., TAYLOR, R. D., CORCORAN, A. C., AND MUELLER, L.: *J. A. M. A.*, **124**: 736, 1944.
7. RUSSEK, H. I., SOUTHWORTH, J. L., AND ZOHMAN, B. L.: *J. A. M. A.*, **128**: 1225, 1945; **130**: 927, 1946.
8. DALE, H. H.: *J. Physiol.*, **34**: 163, 1906.



9. BARGER, C., AND DALE, H. H.: *Biochem. J.*, **2**: 240, 1907.
10. LINGGAR, C. R.: *Am. J. Physiol.*, **129**: 53, 1940.
11. STAHNKE, E.: *Klin. Wochenschr.*, **7** (1): 23, 1928.
12. HAMILTON, W. F., BREWER, G., AND BROTMAN, J.: *Am. J. Physiol.*, **107**: 427, 1934.
13. WILKINS, R. W., AND CULBERTSON, J. W.: *Trans. Assoc. Am. Physicians*, **60**: 195, 1947.
14. FREIS, E. D., STANTON, J. R., MOISTER, F. C., AND WILKINS, R. W.: Unpublished observations.
15. STEAD, E. A., JR., AND EBERT, R. V.: *Arch. Int. Med.*, **67**: 546, 1941.
16. REISER, M. F., AND FERRIS, E. B., JR.: *J. Clin. Investigat.*, **27**: 156, 1948.
17. HINES, E. A., JR., AND BROWN, G. E.: *Am. J. Int. Med.*, **7**: 209, 1933.
18. ROTHLIN, E.: *Bull. Acad. Suisse d. Sci. Med.*, **2**: 1, 1946.
19. BLUNTSCHLI, H. J., AND GOETZ, R. H.: *Am. Heart J.*, **35**: 873, 1948.

# THE DEVELOPMENT OF TOLERANCE IN RATS TO SOME NEW SYNTHETIC ANALGESICS<sup>1</sup>

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The development of tolerance to analgesic action is one of the disadvantages of the repeated use of opiates in the treatment of chronic pain. This makes it necessary to increase the dose which in turn increases the degree of tolerance thereby setting up a vicious cycle. Tolerance has been defined by Himmelsbach (1) as "the gradual decrease in the effect produced by repeated administration of a drug; or, conversely, a gradual increase in the dosage of the drug necessary to produce the same effect as did the initial dose." Most investigators believe that tolerance is intimately associated with the addiction liability of a drug. The development of tolerance to morphine has been investigated in several species of animals and this subject has been comprehensively reviewed by Krueger, Eddy, and Sumwalt (2). The criterion of the development of tolerance to morphine in most animal experiments has been the disappearance of the narcotic effect whereas in clinical practice the decrease in analgesic effect is more important. It has been demonstrated that tolerance to the various actions of morphine develops at different rates and to different degrees.

It has been the goal of the chemists to synthesize a potent analgesic that does not have the disadvantages of morphine. In recent years several new analgesics have appeared. It is of considerable importance that the development of tolerance to their analgesic action be determined. Andrews (3) has reported that in patients, previously addicted to morphine, tolerance develops to the pain threshold raising effects of meperidine (Demerol). By measuring the duration of analgesia, Scott *et al.* (4) found that tolerance develops to this action of methadone in rats. Isbell *et al.* (5) reported that tolerance develops to methadone in mice, dogs and man. By using a radiant thermal stimulus, we have been able to determine the degree of analgesia and the rate of tolerance development in rats to the daily administration of several compounds chemically related to Demerol or methadone. These results are compared with those obtained with morphine.

**METHODS.** Analgesic activity in male albino rats was determined by the Ercoli and Lewis (6) modification of the radiant thermal stimulus method of D'Amour and Smith (7) which measures the reaction time of the animal when subjected to a constant painful stimulus.

We have found that the degree of analgesia can be considered complete when the reaction time of an animal has been increased more than 100 per cent over the time obtained before

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<sup>1</sup> This work is part of an investigation carried out under the supervision of Dr. M. H. Scovors and submitted in a dissertation to the Graduate School of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

medication. Therefore, for purposes of calculation, the increase in reaction time has been calculated as per cent analgesia, the animals not giving a finite reaction time being assigned a value of 100 per cent.

The compounds investigated were morphine sulfate (10 mgm. per kgm. per day), WIN 642-4, *l*-methadone hydrochloride (2 mgm. per kgm. per day), WIN 1783-2, *l*-isomethadone hydrochloride (3 mgm. per kgm. per day), WIN 1539, 1-methyl-4-(3-hydroxyphenyl)-4-piperidylethyl ketone hydrochloride (3 mgm. per kgm. per day) and WIN 1161-2, *l*-ethyl 1,1-diphenyl-3-dimethylaminobutyl sulfone hydrochloride (3 mgm. per kgm. per day). Studies on the analgesic activity of these compounds have been published elsewhere (8-13). The dose of each compound was selected so that the value for the average initial analgesic reaction was between 90 and 100 per cent. Data from a group of 20 rats were obtained for each compound. These rats were given daily subcutaneous injections and their analgesic reactions and body weights determined at weekly intervals.

The administration of the drugs was discontinued at the end of six weeks. At this time, each group was subdivided into three smaller groups. On the second, fourth and seventh days following withdrawal one of these smaller groups was again tested for the analgesic response. On the second and third week after withdrawal the whole group on each compound was again tested for the analgesic response. The groups were divided in this manner so that the time interval between the test doses of the compound for any one animal would be large enough to have as little influence as possible on tolerance. We had previously determined that no tolerance develops when the doses are given at weekly intervals.

**RESULTS.** The results of the analgesic determinations are given in figures 1 and 2. Daily administration of these compounds leads to a progressive decrease in the degree of analgesia produced. There is some difference among the compounds, however, with regard to the degree and rate of development of tolerance. Tolerance developed most rapidly to WIN 1161-2. The analgesic response to this compound was 40 per cent of its initial value within 2 weeks and it remained at this low level until the sixth week when administration was discontinued. The least amount of tolerance developed to WIN 1783-2. At the end of the first week, the response was 85 per cent of its initial value and changed little after this time so that it was still approximately 80 per cent at the end of 6 weeks. The development of tolerance to all the other compounds was quite similar and at the end of 6 weeks of daily administration the analgesic response was found to be only about 40 per cent of the initial value.

It is interesting to note that there was no significant change in the pre-injection reaction time of the animals during the course of the experiment.

Figure 2 shows the time-action curves for the compounds. These indicate that morphine has a greater duration of action than the other compounds. The maximum effect of morphine is reached at 60 minutes following injection whereas the peak effect of the other compounds is obtained at 30 minutes. As tolerance develops there is a decrease in duration of action that is probably related to the decrease in maximum effect.

In addition to the decrease in the analgesic response that results from daily administration of these drugs, there were definite changes in the side-effects. The characteristic side-effects observed with all the compounds, except morphine, with the doses used here are: slight catalepsy, absence of corneal and wink reflexes and exophthalmos. The rats receiving morphine showed a slight depression. After the animals had been medicated for 1 week, the most charac-

teristic side effects were nervousness and excitability which made their handling more difficult. The depression caused by morphine disappeared after 3 weeks of administration and was replaced by excitation. These effects were observed after each daily injection and continued for the duration of the experiment. The

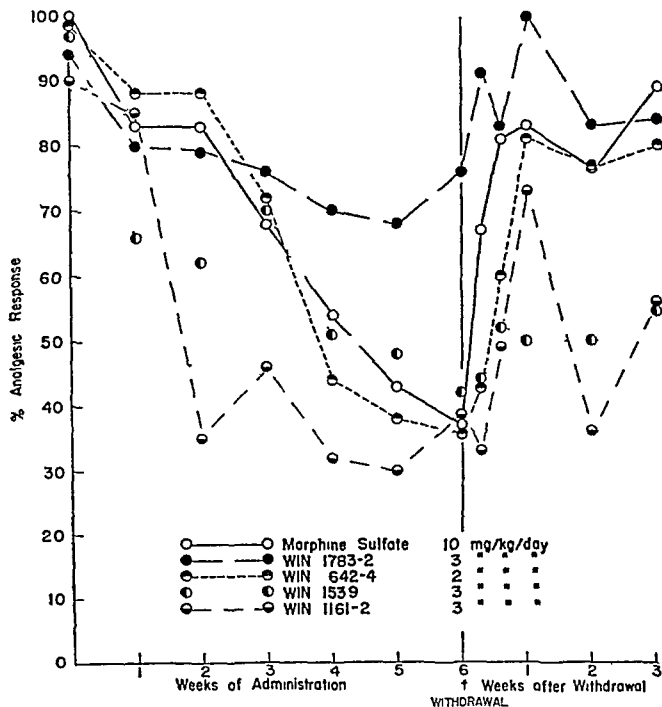


FIG 1 EFFECT OF DAILY ADMINISTRATION ON THE ACTION OF SOME ANALGESIC COMPOUNDS

excitation occurred within about 30 minutes after injection and lasted for about 2 hours.

The rates of recovery from the tolerance after withdrawal of these compounds are given in figure 1. The fact that smaller numbers of animals were used for the first 3 tests after withdrawal probably accounts for part of the variations observed in some of these responses. At the end of 3 weeks after withdrawal, none of the group had recovered its original analgesic response, although there

were observed differences in the degree of recovery with the various compounds. WIN 1783-2, which showed the least amount of tolerance, gave 90 per cent of the initial response at the end of 3 weeks after withdrawal. Of the compounds developing the greatest degree of tolerance, the most rapid recovery was observed with morphine and WIN 642-2, these giving responses of 90 and 82 per cent of the normals, respectively, at the end of 3 weeks after discontinuance of medication. Compounds WIN 1539 and WIN 1161-2 showed the least recovery for

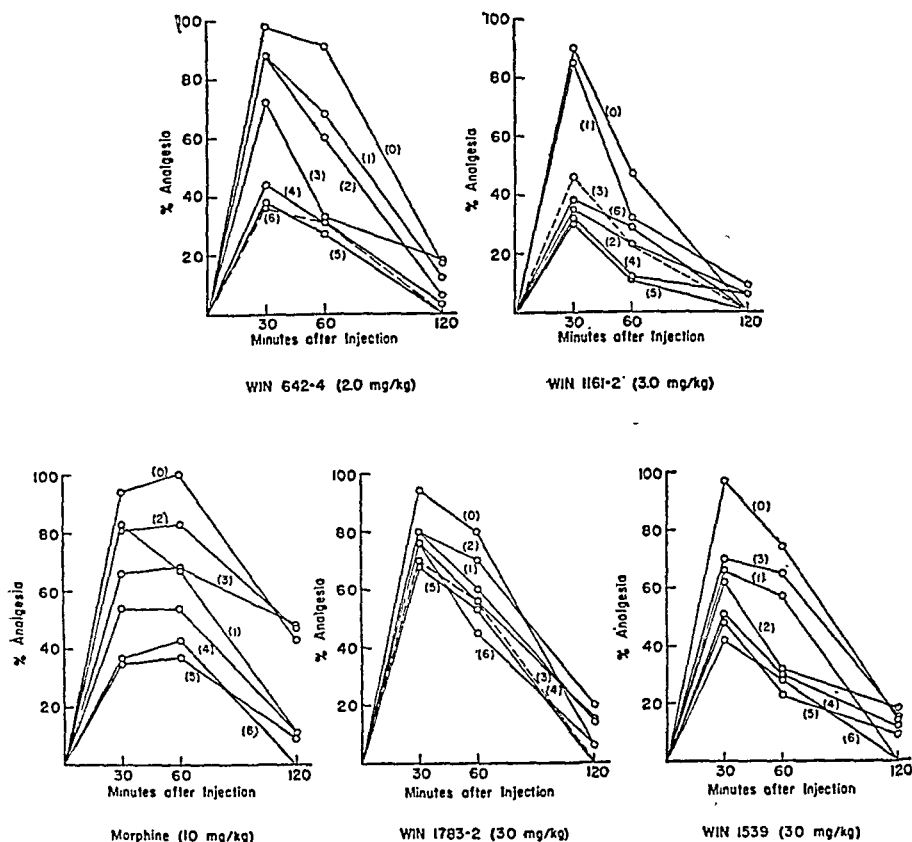


FIG. 2. TIME-ACTION CURVES OF ANALGESICS DURING DEVELOPMENT OF TOLERANCE. Numbers in parentheses indicate week of determination (0 = initial)

3 weeks after withdrawal. WIN 1161-2 gave a response of about 62 per cent of normal and WIN 1539 about 57 per cent.

After withdrawal of the drugs, the animals were observed for possible abstinence symptoms. No symptoms were observed at 24 hours, but at 48 hours after withdrawal the animals exhibited a marked degree of excitation as evidenced by increased activity, fighting and biting one another and by chewing on the wires of the cages. This excitation corresponded to that which was observed following the daily injection of the compound during the test period.

At 96 hours there was still some excitation present but it was less than that observed at 48 hours. This excitation was increased after the injection of the drugs during the withdrawal period. Two weeks after withdrawal, the group receiving morphine still developed a marked degree of excitation following injection. A slight excitation was observed in animals receiving WIN 1539 and WIN 1161-2. With WIN 642-4 and WIN 1783-2, slight catalepsy, absence of corneal reflex, exophthalmos and only a very slight excitability was observed. Essentially the same effects were observed 3 weeks following withdrawal.

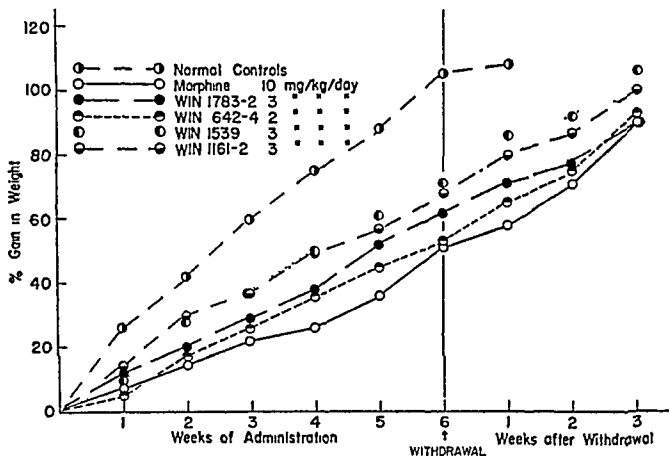


FIG 3 EFFECT ON WEIGHT GAIN OF DAILY ADMINISTRATION OF SOME ANALGESIC COMPOUNDS

In order to determine whether or not cross tolerance had developed between WIN 1539 and morphine, 10 of the animals which were tolerant to the action of WIN 1539 22 days following withdrawal were given 10 mgm per kgm of morphine. There was an indication that little or no cross tolerance had developed from WIN 1539 to morphine. The cross tolerance from morphine to WIN 1539 was not determined.

The percentage gains in weight are given in figure 3. There was a decreased rate of growth in all groups receiving daily drug injections. Although it was not measured in this experiment, we believe this is due in part to a decreased consumption of food. Hoppe (14) found a decreased food consumption with rats in a subacute toxicity study of methadone. Even after withdrawal of the drug there was no appreciable change in the rate of growth. Morphine caused the greatest and WIN 1539 the least depression of growth.

DISCUSSION. Scott *et al.* (4) and Isbell *et al.* (5) have reported the development of tolerance to the analgesic action of methadone, one of the newer synthetic analgesic drugs described by Kleiderer *et al.* (8). We have determined the effect of the daily administration to rats of 3 compounds in the methadone series and one related to Demerol. Whereas Scott found a decrease in the duration of analgesia produced by methadone following repeated administration in rats, Isbell found that the tolerance developed to the analgesic action of methadone in mice was of the same degree as that produced by morphine but that there was no material change in the duration of action. The results we have obtained with *l*-methadone were very similar to morphine, both in the degree of tolerance developed and in the decrease of the duration of action.

The tolerance developed to the action of *l*-isomethadone is significantly less than that of *l*-methadone. This marked difference between the 2 compounds is particularly interesting in view of the report by Isbell and Eisenman (15) on the addiction liabilities of *l*-methadone and isomethadone. These workers found that larger doses of isomethadone than of *l*-methadone were required to reduce the abstinence symptoms of morphine and that isomethadone, in the doses used, was not an entirely adequate substitution for morphine in addicts, whereas *l*-methadone was adequate.

The most rapid development of tolerance was observed with the compound in which the ketone group of *l*-methadone had been replaced by a sulfone group (WIN 1161-2).

We observed a marked change in the nature of the side-effects during the development of tolerance. After a week of daily administration of the compounds the animals showed a marked degree of excitation following each injection indicating the rapid development of tolerance to the depressant action. Joel and Ettinger (16) also observed in rats that during the development of tolerance to morphine the initial narcotic phase disappeared and excitation became prominent. Tatum, SeEVERS and COLLINS (17) reported that the excitation phase of morphine action masked the depressant phase in the tolerant state. The compounds tested in this experiment did not differ essentially from morphine in this respect.

The only symptom seen following withdrawal of the compounds was a hyperexcitability which reached its maximum at 48 hours following withdrawal and then became progressively less. We did not see the increase in pre-injection hyperirritability during the daily administration of the compounds that Himmelsbach, Gerlach and Stanton (1) found in rats receiving morphine and which they interpreted as evidence of addiction.

#### SUMMARY

1. We have demonstrated the development of tolerance in rats to the analgesic action of morphine and several new analgesic drugs by measuring the degree of analgesia to a standard radiant thermal stimulus.

2. At the end of 6 weeks of daily subcutaneous administration of morphine (10 mgm. per kgm. per day), WIN 642-4, (2 mgm. per kgm. per day), WIN

1539 (3 mgm. per kgm. per day), and WIN 1161-2 (3 mgm. per kgm. per day), the analgesic response was less than 50 per cent of the initial value. The response of WIN 1783-2 (3 mgm. per kgm. per day) at the same time was about 80 per cent of normal.

3. After discontinuance of the drugs, rats treated with WIN 1539 and WIN 1161-2 showed the least amount of recovery from the tolerance. With the other compounds, responses of about 90 per cent of normal were obtained at 3 weeks after withdrawal.

4. There was a change in the character of the side-effects from depression to excitation following drug injection during the development of tolerance.

5. After withdrawal, the only symptom observed was hyperexcitability which reached its maximum in about 48 hours.

6. The daily administration of these compounds caused a decreased rate of growth which was probably due to a decrease in food consumption.

7. Of the compounds tested, *L*-isomethadone showed the least tendency to develop tolerance and to induce the addiction syndrome, as it is seen in rats.

ACKNOWLEDGMENT. The author wishes to express his sincere appreciation to Mr. John Frick and Miss Ora Hoeltzel for their technical assistance in this work.

#### REFERENCES

1. HIMMELSBACH, C. K., GERLACH, G. H., AND STANTON, E. J.: *THIS JOURNAL*, 53: 179, 1935.
2. KRUEGER, H., EDDY, N. B., AND SUMWALT, M.: *The Pharmacology of the Opium Alkaloids*. Public Health Reports, Supplement No. 165.
3. ANDREWS, H. L.: *THIS JOURNAL*, 75: 333, 1942.
4. SCOTT, C. C., CHEN, K. K., KOHLSTAEDT, K. G., ROBBINS, E. B., AND ISRAEL, F. W.: *THIS JOURNAL*, 91: 147, 1947.
5. ISBELL, H., WIKLER, A., EDDY, N. B., WILSON, J. L., AND MORAN, C. F.: *J. A. M. A.*, 135: 888, 1947.
6. ERCOLI, N., AND LEWIS, M. N.: *THIS JOURNAL*, 84: 301, 1945.
7. D'AMOUR, F., AND SMITH, D. L.: *THIS JOURNAL*, 72: 74, 1941.
8. KLEIDERER, E. C., RICE, J. B., CONQUEST, V., AND WILLIAMS, J. H.: Report No. 981, Office of the Publication Board, Department of Commerce, Washington, D. C.
9. SCOTT, C. C., KOHLSTAEDT, K. G., AND CHEN, K. K.: *Current Res. Anesth. and Analg.*, 26: 12, 1947.
10. THORP, R. H., WALTON, E., AND OFNER, P.: *Nature*, 160: 605, 1947.
11. LUDUENA, F. P., MILLER, L. C., ANANENKO, E., AND FRICK, J. D.: *Fed. Proc.*, 7: 241 1948.
12. JENNY, E. N., AND PFEIFFER, C. C.: *Fed. Proc.*, 7: 231, 1948.
13. KLENK, M. M., SUTER, C. M., AND ARCHER, S.: *J. Am. Chem. Soc.*, 70: 3846, 1948.
14. HOPPE, J. O.: To be published.
15. ISBELL, H., AND EISENMAN, A. J.: *THIS JOURNAL*, 93: 305, 1948.
16. JOEL, E., AND EPTINGER, A.: *Arch. f. exper. Path. u. Pharmacol.*, 116: 334, 1926.
17. TATUM, A. L., SCYVERS, M. H., AND COLLINS, K. H.: *THIS JOURNAL*, 36: 446, 1929.



# THE GANGLIONIC BLOCKING ACTION OF "DIBUTOLINE"

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In 1943 Swan and White (1) studied a series of choline esters with atropine-like properties. The dibutyl urethane of choline, and a closely related compound, the dibutyl urethane of dimethylethyl- $\beta$ -hydroxyethyl ammonium sulfate, were selected for clinical trial as mydriatic and cycloplegic agents. In a pharmacological study of the actions of the latter compound ("Dibutoline"), Peterson and Peterson (2) demonstrated blockade of the actions of acetylcholine and parasympathetic nerve stimulation on cholinergic effector organs, but stated that the drug possessed no "nicotinic paralyzing" action. Some of the characteristics of the cardiovascular responses to Dibutoline, however, suggested that blockade of autonomic ganglia was produced by the drug. In dogs anesthetized with pentobarbital Dibutoline caused a decrease of heart rate not affected by atropine or by vagotomy, and a fall of blood pressure limited in extent. Both are characteristic features of the cardiovascular actions of tetraethylammonium salts (3). The following experiments were planned to determine whether Dibutoline possesses ganglionic blocking activity in addition to its atropine-like action.

**METHODS.** Experiments were performed on dogs and cats anesthetized with Dial<sup>1</sup> or with thiopental and barbital. Arterial pressure was recorded with a mercury manometer in dogs and with a membrane manometer in cats. Femoral arterial blood flow was recorded with a simple differential manometer (4). Contraction of the cat's nictitating membrane was induced by pre- and post-ganglionic stimulation of the cervical sympathetic nerve. Pre- and post-ganglionic stimulation of cardiac sympathetic nerves was performed in one experiment, and heart rate was recorded by means of an electrocardiograph. Dibutoline<sup>3</sup> was used as a 1 per cent solution; all doses are in terms of the sulfate.

**RESULTS.** *Arterial pressure and blood flow.* The depressor and cardiodecelerator actions of Dibutoline were compared with those of tetraethylammonium (T.E.A.) in 2 dogs. Intravenous injection of Dibutoline, like T.E.A., caused a fall of pressure and a decrease of heart rate accompanied by an increased blood flow in the femoral artery (fig. 1). Nearly maximal effects were obtained with 1 mgm./kgm. of Dibutoline, as compared with about 1.5 mgm./kgm. of tetraethylammonium chloride<sup>3</sup>. Since agents which cause vasodilatation by a direct action on arteriolar smooth muscle usually diminish the femoral blood flow as the pressure falls, these effects of Dibutoline suggested a neurogenic mechanism. Intraarterial injection of Dibutoline, however, caused a slight but significant increase of blood flow; but intraarterial injection of a control solution of 0.25 per

<sup>1</sup> Supported by a grant from the Life Insurance Medical Research Fund.

<sup>2</sup> United States Public Health Service Fellow.

<sup>3</sup> Dial was supplied by the Ciba Laboratories, Dibutoline by Merck and Co., and tetraethylammonium chloride as "Etamon" by Parke, Davis & Co.

cent phenol (used as a preservative in the solution of Dibutoline) caused comparable flow changes, and it is probable that Dibutoline itself has little or no direct vasodilator activity. Dibutoline injected intravenously during the hypotension induced by continuous infusion of T E A caused no further fall in

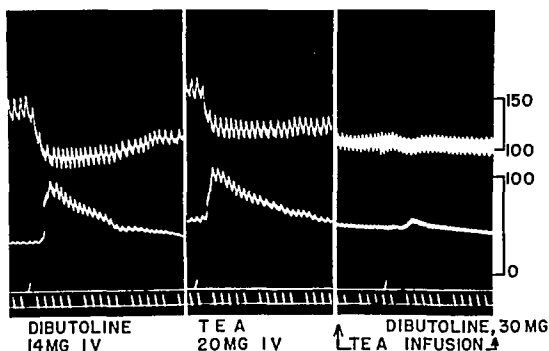


FIG 1 Dog, 14.5 kgm, thiopental barbitol anesthesia. Tracings, top to bottom carotid arterial pressure (scale at right in mm Hg), femoral arterial blood flow (scale at right in cc/min), signal, time in 10 sec intervals. All doses administered intravenously.

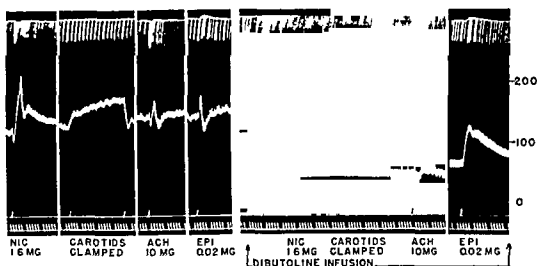


FIG 2 Dog, 16 Kg, thiopental and barbitol. Tracings from top to bottom respiration, arterial pressure (scale at right), signal, time. Previous to first segment, atropine, 0.5 mgm/kgm. Nic = nicotine tartrate, Ach = acetylcholine chloride, Epi = epinephrine hydrochloride. Dibutoline infusion, 20 mgm/kgm/hr following priming dose of 3 mgm/kgm.

pressure, although the depressor effect of an agent causing peripheral vasodilatation by a direct action on the vessels should be potentiated by ganglionic blockade (5).

*"Paralyzing nicotinic" action* T E A prevents the pressor effects of carotid occlusion and the pressor and hyperpneic responses to nicotine and large doses of acetylcholine, and, by eliminating compensatory reflexes, potentiates the

pressor action of epinephrine. Dibutoline was shown to duplicate these actions in the experiment illustrated in figure 2. After the administration of atropine, 0.5 mgm./kgm., control responses were obtained to epinephrine, carotid clamping, nicotine (1.6 mgm.), and acetylcholine (10 mgm.). Dibutoline was injected in a priming dose of 3 mgm./kgm. followed by continuous infusion at a rate of 20 mgm./kgm./hr. in order to maintain a completely blocking level of the drug. During the infusion, carotid clamping failed to elevate pressure; the pressor responses to epinephrine were potentiated, and the actions of nicotine and acetylcholine were abolished (fig. 2). Two hours after the infusion was discontinued recovery had occurred, and all responses were restored nearly to their original levels.

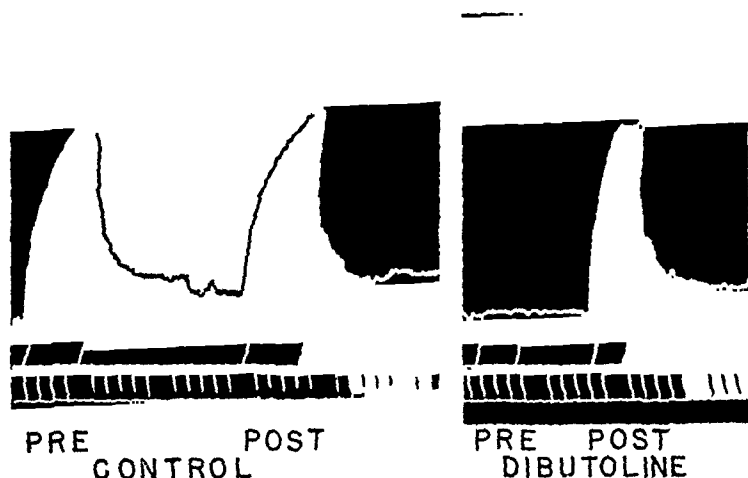


FIG. 3. Cat, 2.5 Kgm., Dial anesthesia. Response of nictitating membrane to brief periods of pre- and postganglionic stimulation of the cervical sympathetic, before and after Dibutoline, 2 mgm./kgm.

*Blockade of sympathetic ganglia.* In 2 cats under Dial anesthesia, pre- and post-ganglionic connections of the superior cervical ganglion were exposed, and the nictitating membrane was attached to a recording lever. Contractions of the membrane were produced by brief periods of stimulation of the preganglionic and postganglionic fibers. Dibutoline in a dose of 5 mgm. abolished the effect of preganglionic, but not of postganglionic stimulation and did not alter the contractile response to 10 microgm. of epinephrine (fig. 3).

In a dog anesthetized with thiopental and barbital, the chest was opened, the vagi were cut, and the preganglionic connections of both stellate ganglia were crushed as far from the ganglia as possible. Heart rate was recorded with the electrocardiograph during preganglionic and postganglionic stimulation of the accelerator nerves. Dibutoline, like T.E.A., blocked the effects of preganglionic, but not postganglionic, stimulation.

**DISCUSSION.** The experiments clearly demonstrate that Dibutoline possesses a ganglionic blocking action in addition to its atropine-like action. This activity

explains the action of the drug on the heart rate of animals under barbiturate anesthesia, and accounts for most if not all of its depressor action. The drug prevents the pressor (i.e., "nicotinic stimulating") actions of nicotine and of acetylcholine. It is likely that suppression of the ganglionic stimulating action of these agents is competitive in nature, i.e., that larger doses of nicotine and acetylcholine would still have provoked pressor reactions. This was not tested. Since the atropine-like action of Dibutoline is demonstrable with doses much smaller than those necessary to block ganglia, it should certainly be possible to "unmask" the pressor action of acetylcholine, as stated by Peterson and Peterson (2), with doses which fail to abolish the pressor action of acetylcholine.

Because of the high doses of Dibutoline required to block ganglia, as compared with its relatively potent atropine-like activity, it is not likely that the actions demonstrated in this study play any significant part in the human response to the drug when used clinically to produce mydriasis or relaxation of intestinal smooth muscle. Only the highest doses reported in human subjects, about 30 mgm subcutaneously (6), would be expected to cause effects on ganglionic transmission.

#### SUMMARY

Dibutoline, in addition to its atropine-like action, produces effects comparable with those of tetraethylammonium: (1) fall of heart rate and blood pressure with increased femoral blood flow, (2) abolition of the pressor response to carotid occlusion, (3) suppression of the pressor actions of nicotine and acetylcholine, (4) potentiation of the pressor effects of epinephrine. These actions were shown to be due to blockade of ganglionic transmission.

#### REFERENCES

- 1 SWAN, K. C., AND WHITE, N. G. *Proc Soc Exper Biol and Med*, **53**: 164, 1943
- 2 PETERSON, C. G., AND PETERSON, D. R. *THIS JOURNAL*, **84**: 236, 1945
- 3 ACHESON, G. H., AND MOE, G. K. *THIS JOURNAL*, **87**: 220, 1946
- 4 MOE, G. K. *Science* (In press)
- 5 MOE, G. K. *J A M A*, **137**: 1115, 1948
- 6 PETERSON, C. G., AND PETERSON, D. R. *Gastroenterology*, **5**: 169, 1945

# PHARMACOLOGIC AND TOXICOLOGIC STUDIES ON DIETHYL-AMINOETHYL ESTER OF 1-PHENYL-CYCLOPENTANE-1-CARBOXYLIC ACID HYDROCHLORIDE, "PARPANIT"<sup>1</sup>

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The diethylaminoethyl ester of phenyl-cyclopentane-carboxylic acid, as the hydrochloride, "Parpanit", has had extensive clinical trial in Europe in the treatment of Parkinsonism and similar disorders. Domenjoz (1) noted that Parpanit dilates the blood vessels in the isolated rabbit ear, and that large doses stimulate the central nervous system in rats. It antagonizes the effects of acetylcholine and of barium chloride on the rabbit intestine and of acetylcholine on rabbit and guinea pig uterus. He further observed a decrease in the blood pressure in rabbits, cats and dogs following the injection of Parpanit and an action on the frog nerve-muscle preparation analogous to that of curare.

Heymans and de Vleeschhouwer (2) confirmed the hypotensive action of Parpanit in the dog and described the mechanism as a peripheral vasodilatation independent of the central nervous system. They found moderate doses of Parpanit to depress the inhibitory effects of the vagus nerve on the heart, and to evoke, after a very brief tachycardia, a fairly marked bradycardia, despite the paralysis of the vagal reflexes. Larger doses were necessary to block the cardio-inhibitory action of acetylcholine, although its hypotensive component was never eliminated by any dosage. An intravenous dose of Parpanit caused immediate inhibition of respiration but this inhibition was followed by a long period of stimulation. Hyperperistalsis of the gut and spasm of the bronchi induced in dogs by appropriate drugs could be reduced by Parpanit. Voluntary muscle responses and neuromuscular conduction were unaffected by massive treatment with the drug, although fasciculation induced by diisopropyl fluorophosphate, nicotine or acetylcholine was either depressed or suppressed.

Charlier and Philippot (3), in experiments on human subjects, found that the inhalation of Parpanit vapors produced a marked dilatation of bronchial smooth muscle and relieved the dyspnea induced by the inhalation of carbaminoylecholine. Lechner and Lanini (4) experimented with solutions of various strengths and noted a stimulation of the metabolism of rat muscle slices by Parpanit concentrations of 1:100,000 with a gradual decrease in effect until at 1:35,000 inhibition appeared and this depression increased as the concentrations rose.

In view of the drug's extensive use in Europe and the inauguration of clinical trials in this country, it was deemed important to extend the work of previous

<sup>1</sup> This investigation was made possible through a grant by the Geigy Company, Inc. for research in science.

investigators in a thorough pharmacologic and toxicologic study of Parpanit, including a comparison with trasentine<sup>2</sup> and atropine

**Acute Toxicity** The LD<sub>50</sub>'s were determined using a minimum of 26 animals for each dose level with the exception of one group of intravenously-injected mice which numbered 17. The results were projected logarithmically and the LD<sub>50</sub>'s derived as summarized in table 1. The intravenous injections were made at a constant rate, the mice receiving 1.68 mgm of Parpanit per minute in 0.3 per cent solution and the rabbits 18.4 mgm of the drug per minute in a 2.0 per cent solution. As can be seen in the table the drug has a relatively low order of toxicity and is more toxic when given intravenously than when administered intraperitoneally. The figures cited here for LD<sub>50</sub> differ from the "average lethal dose" of Domenjoz (1) for intravenously administered Parpanit in rabbits of 12 mgm/kgm and in mice of 67.5 mgm/kgm. It is impossible to resolve the apparent discrepancies since the definition of the term and the data on animal

TABLE 1  
*Acute toxicity of Parpanit*

ANIMAL	NO OF ANIMALS	AVERAGE WEIGHT AND RANGE	ROUTE OF ADMINISTRATION	LD <sub>50</sub> MG/ KG
Rabbits	73	2.24 kgm (1.77-2.92 kgm)	Intravenous	24.5
Mice	50	20 gm (13-30 gm)	Intravenous	45.1
Mice	90	17 gm (14-23 gm)	Intraperitoneal	222.3
Rats	147	136 gm (101-168 gm)	Intraperitoneal	209.0
Cats	27	2.73 kgm (1.9-4.2 kgm)	Oral	390

weight, concentration of solutions and rate of injections are not available for Domenjoz' determinations.

Large doses of Parpanit in mice, rats and rabbits gave evidence of a marked stimulation of the central nervous system. In rats and mice toxic but sublethal doses evoked progressively increasing generalized hyperactivity, jumping, clamping of jaws, rapid pawing with the front legs while sitting erect on the hind legs, clonic convulsions, followed by lethargy or coma. With increase in the dosage, convulsions appeared rapidly with a minimal incidence of the lesser symptoms and were followed by coma, with respiratory difficulties and sometimes death. During recovery from coma convulsive movements often appeared. Intravenous injections in rabbits of large amounts of Parpanit led to weakness of the muscles of the extremities, clonic convulsions followed by either coma or death. Death appeared to result from respiratory paralysis in all three species of animals.

**Tolerance and Chronic Toxicity** A group of 20 albino male rats (average weight

<sup>2</sup> Trasentine obtained from the Ciba Pharmaceutical Products, Inc. through the courtesy of Dr. Frederick F. Lonkman.

163.3 gm.) was injected daily except Sundays for 2 weeks with 100 mgm. of Parpanit per kgm. intraperitoneally. The first injection induced convulsions in 17 of the 20 animals and subconvulsive excitement in one. Convulsions resulted from the daily injections in most animals throughout the period of treatment and following the last injection on the 12th day, 14 of the 17 survivors reacted with convulsions. Of the 20 rats, 3 died during the period of the experiment, none of these, however, during a period of several hours immediately following an injection. The 17 rats which survived the experiment weighed an average of 169.9 gm. at the beginning and 159.9 gm. at the end. Autopsy of animals dying during the period of injection and of some killed at the end of the experiment revealed no gross pathology. A number of rats were still alive and in good health more than 4 months after the end of the experiment.

*Cardiovascular System and Respiration.* A series of 13 dogs was used for the studies on blood pressure and respiration. With the animals under light ether anesthesia arterial pressure was recorded by means of a mercury manometer from a cannula in the right carotid artery with heparin as an anticoagulant. Respiration was recorded in most experiments by means of a tambour connected to a pneumograph about the chest of the dog. Parpanit and trasentine were injected intravenously in doses of 0.5 to 5.0 mgm./kgm. and 0.2 to 2.0 mgm./kgm., respectively. These injections were made rapidly since slow injections were found to have very little effect upon the blood pressure.

Parpanit and trasentine were found to produce a drop in arterial pressure, which increases in intensity and duration as the dosage and speed of injection increase, thus confirming the report of Heymans and de Vleeschhouwer (2). The magnitude of the drop was not influenced by previous injections of the drugs under study and only slightly altered by differences in the control arterial tension, the highest pressures (over 160 mm. Hg) showing a slightly greater response and the lowest (under 100 mm. Hg) a slightly lower response.

Electrocardiographic studies using Lead II were made on 4 dogs, 2 trained unanesthetized and 2 under morphine-urethane anesthesia. Blood pressure tracings from the carotid arteries of the latter were correlated with the electrocardiographic findings. All 4 animals received first 1.0 mgm./kgm. of Parpanit intravenously and this was followed after 10 minutes in the 2 unanesthetized animals by 2.0 and 3.0 mgm./kgm., making a total of 3.0 and 4.0 mgm./kgm. in these animals, respectively. No alterations in the electrocardiographic pattern could be detected in any of the animals with these doses of Parpanit. The heart rate rose abruptly during the injection to nearly double that of the control and, though it gradually declined, remained well above the control rate for half an hour or more. Neither section of the vagus nor the second and larger injection in the 2 anesthetized animals further increased the rate. The persistent tachycardia is a surprising contrast to the early and definite bradycardia reported by Heymans and de Vleeschhouwer (2) following doses of Parpanit adequate to paralyze the vagus in the heart. These workers used chloralose as their anesthetic while the observations here reported were made on animals either unanesthetized or under morphine-urethane anesthesia.

The paralysis of the vagal cardio-inhibitory fibers (2) was confirmed in 12 experiments on 7 dogs in which the right vagus was stimulated with a faradic current before and after the injection of Parpanit. The antagonism by Parpanit to the cardio inhibitory but not to the hypotensive action of acetylcholine (2) was confirmed in 21 experiments on 6 dogs. It was further noted that the effectiveness of Parpanit in inhibiting acetylcholine depended on the relative amounts of Parpanit and acetylcholine administered. The results in figure 1 are typical of all of these experiments. At 5, 3 mgm /kgm of Parpanit blocked the vagus and eliminated the cardio inhibitory action of 1 mgm of acetylcholine in this dog but was inadequate to block the action of 4 mgm of acetylcholine at 6. A further

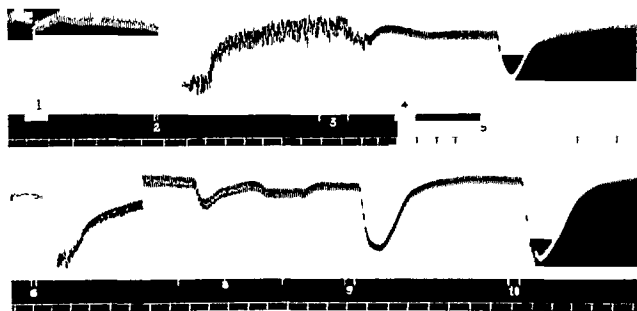


FIG 1 Dog ♀ 16 kgm Ether anesthesia. Top curve is that of the arterial blood pressure with a mercury manometer, middle curve, the time and duration of stimulation of the vagus nerve stimulated electrically, 2 Intravenous injection of Parpanit 3 mgm /kgm total, 3 Intravenous injection of Parpanit 3 mgm /kgm, 4 Vagus nerve stimulated electrically, 5 Acetylcholine chloride 1 mgm, 6 Acetylcholine chloride 4 mgm, 8 Parpanit 5 mgm /kgm, 9 Acetylcholine chloride 4 mgm, 10 Acetylcholine chloride 8 mgm

injection of 5 mgm /kgm of parpanit at 8, was sufficient to Prevent the cardio-inhibitory effect of 4 mgm of acetylcholine at 9, but not that of 8 mgm at 10

Fifteen experiments in which the splenic volume was recorded by means of an *oneometer* connected to a modified Brodie bellows generally showed a brief decrease in volume corresponding to the decline in blood pressure, although in 4 of the 5 experiments with a small dose of 0.5 mgm /kgm of Parpanit, there was some increase in volume of the organ

The amplitude of respiration was initially depressed by Parpanit in 17 of the 19 experiments. Following the brief hypotensive phase, slowing of the respiratory rate was observed and a gradual increase in the amplitude above the normal was seen. As these experiments on etherized dogs did not show the respiratory stimulation described by Heymans and de Vleeschhouwer (2) who used dogs under chloralose anesthesia, it is possible that the control respiration was basically



different in the 2 groups of animals due to the different anesthetics employed. Death from toxic amounts of Parpanit is apparently due to respiratory failure.

**Turtle Heart.** The turtle heart was used to determine the action of Parpanit on vagal conduction to the cold-blooded heart. With the heart arranged *in situ* for recording contractions, solutions of the drugs were applied in a pocket of the pericardium surrounding the heart for a period of 10 minutes. The right vagus nerve was stimulated for a period of 20 seconds at 2-minute intervals by a faradic current derived from a Harvard inductorium. This current was adequate to inhibit the heart throughout the 20 seconds of excitation of the nerve. The efficacy of a drug in inhibiting the vagus action was indicated by the number of "escapes" of the heart during the period of stimulation. Sixty-four experiments were performed with Parpanit, 41 with trasentine and 41 with atropine.

All 3 drugs were found to be active in paralyzing the cardiac vagus nerve of the turtle, atropine showing the greatest activity and trasentine the least. The ratio for approximately equal effects was atropine 1:Parpanit 20:trasentine 50.

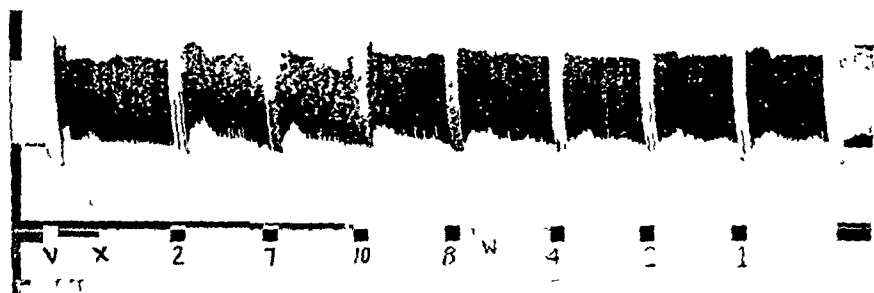


FIG. 2. Turtle heart *in situ*. Top curve is that of ventricular contractions, up stroke systole, down stroke diastole; middle line, vagal stimulation ("V" control, and figures showing number of "escapes"); bottom line, time in intervals of 20 seconds. X—application of Parpanit 1:500,000 in Ringer's solution to outside of heart. W—wash.

Parpanit showed activity in 6 of the 12 experiments at a dilution of 1:1,000,000 and 14 of the 16 at 1:500,000 one of which is shown in figure 2. In this figure from X to W the heart was being bathed with a 1:500,000 Parpanit in Ringer's solution. At V the right cardiac vagus nerve was excited electrically before the application of Parpanit to the heart and complete inhibition of the heart is observed during the 20 seconds of excitation. Two minutes after the drug was applied, at 2, two contractions of the heart occurred during stimulation of the cardiac vagus nerve. Four minutes after the drug was applied, 7 contractions occurred during stimulation, at 7, and two minutes later, at 10, ten contractions. At W the heart was washed and bathed with Ringer's solution and a gradual and complete recovery of the vagus nerve may be noted. Atropine was effective in 10 of the 13 experiments with a 1:10,000,000 solution. The duration of action of atropine considerably exceeded that of the other drugs.

**Frog Heart.** The actions of Parpanit, trasentine and atropine on the contractions of ventricular muscle were studied on 32 frog hearts. A modified Straub cannula was used for perfusion. The ventricle was perfused, usually for 3 minutes, with the drug in Ringer's solution following which the solution was replaced with

fresh Ringer's In this manner a total of 110 experiments was done with Parpanit, 74 with trasentine and 65 with atropine

Parpanit in concentrations of 1:500,000 and higher regularly decreased the amplitude of contractions of the frog ventricle With a solution of 1:25,000, marked toxicity was observed, 6 of the 35 experiments resulted in a complete cessation of contractions The rate of contractions was little affected until a concentration of 1:50,000 was reached, whereupon 19 of the 26 experiments resulted in decreases On the basis of this series of experiments, Parpanit appears to have the greatest activity in depressing the frog ventricle, about 10 times that of atropine and approximately one-third greater than that of trasentine

*Guinea Pig Lung* Thirty-two pairs of excised guinea pig lungs, perfused by the method of Sollman and von Oettingen (5) with a solution described by Tainter, Pedden and James (6), were used in these studies The degree of constriction of the bronchial tree was reflected in the rate of flow measured by means of a sensitive tambour which registered each bubble of air entering the Mariotte bottle containing the perfusion solution

Prior to each experiment, the rate of flow through the preparation was standardized at  $45 \pm 5$  bubbles per minute by adjusting the height of the Mariotte bottle If a pressure of over 30 cm of water was required to maintain the standard perfusion flow, the preparation was discarded At the end of each experiment, a rapid flow was established by the administration of 0.1 mgm of epinephrine hydrochloride, the preparation was thoroughly flushed by a period of rapid perfusion and used for another experiment

The first application of either Parpanit or trasentine but not of atropine, to a fresh preparation, even in small quantities, provoked a gradual and strong constriction, which proceeded to complete cessation of flow in 3 to 4 minutes This spasm could be prevented or relieved by the administration of epinephrine Atropine failed to antagonize this spasm In view of its invariable appearance, the spasm induced by the initial infusion of Parpanit or trasentine is probably not the same as the spasm which occasionally appears spontaneously in an untreated preparation when perfusion is begun Following the initial spasm of the preparation, subsequent injections of Parpanit, trasentine or atropine neither increased nor decreased the rate of flow These drugs were compared as to their potency in preventing the spasm induced by acetylcholine and histamine The antispasmodic was introduced into the perfusate and followed in 40 seconds by a similar injection of 1.0 mgm of acetylcholine chloride or 0.025 mgm of histamine acid phosphate These amounts generally produce total constriction in an untreated lung Since any flow which persisted following treatment with acetylcholine or histamine was a measure of the inhibition of the drug previously applied, this potency is expressed in table 2 as the ratio of the perfusion rate following the bronchoconstrictor drug to the rate before its application

All 3 drugs were effective in inhibiting the action of acetylcholine and to a lesser degree that of histamine, atropine being most active in the amounts used Parpanit and trasentine were essentially similar, with Parpanit probably less effective against histamine

The action of atropine was considerably longer than that of Parpanit or of

trasentine in most instances inhibiting the response to acetylcholine for 20 minutes while the inhibitory effect of Parpanit and trasentine was dissipated in 5 minutes in most experiments.

*Intact Intestine.* These experiments were performed on 7 trained unanesthetized dogs, 4 with Thiry-Vella loops and 3 with modified Mann loops of the small intestine. The latter were prepared by sectioning the intestine at the desired level and bringing the cut end of the caudal portion of the gut to the outside through a stab wound. The cut end of the cephalic portion was closed with a purse-string suture. This end of the gut was connected by a side-to-side anastomosis with a large stoma to the caudal section of intestine about 20 to 25 cm. below its opening to the outside of the abdomen. In using this preparation,

TABLE 2  
*Inhibition of bronchoconstriction*

ANTISPASMODIC DRUG	AMOUNT	BRONCHOCONSTRICTOR DRUG	AMOUNT	NO. OF EXPERIMENTS	INHIBITION PER CENT	
					Range	Mean
	cc.		mgm.			
Atropine	1	Acetylcholine	1	3	64.8-92.6	77.4
Atropine	0.5	Acetylcholine	1	6	24.5-86.7	72.8
Atropine	0.25	Acetylcholine	1	6	51.1-91.1	79.8
Parpanit	1	Acetylcholine	1	19	20.8-95.7	62.0
Parpanit	0.5	Acetylcholine	1	9	16.7-91.2	57.5
Trasentine	1	Acetylcholine	1	13	31.9-93.8	64.0
Trasentine	0.5	Acetylcholine	1	9	13.9-86.7	57.5
Atropine	1	Histamine	.025	5	21.3-88.8	55.9
Parpanit	1	Histamine	.025	9	0 -42.5	24.3
Trasentine	1	Histamine	.025	8	21.6-88.8	42.4

Antispasmodics in 1:1,000 solution in 0.9 per cent sodium chloride solution were administered 40 seconds prior to application of constrictor drug. Perfusion rate stabilized prior to each experiment at  $45 \pm 5$  air bubbles into Mariotte bottle per minute.

the balloon was inserted sufficiently far into the intestine to lie in the normally functioning section. The recording technique has been described by one of us (7).

Twenty-six experiments were carried out with Parpanit in intravenous doses of 0.25 to 1.0 mgm./kgm., 20 with trasentine 1.0 to 3.0 mgm./kgm., and 14 with atropine in a constant dose of 0.05 mgm./kgm. There was a decline in the control activity of the Thiry-Vella loops as time went on, but no differences in drug response could be detected between these and the normally functioning intestine recorded through the Mann loops. Animals were used at intervals of one week with rotation of the drugs in all dogs.

Figure 3, typical of the results with all 3 drugs, is the record from an experiment with Parpanit in a dog with a Thiry-Vella loop. In this figure, at 1, 0.5 mgm./kgm. of Parpanit was injected intravenously. As a result both the general tonus and the height of the rhythmical contractions were markedly diminished. Since the recording apparatus was the same for all drugs, measurement of the

decrease in height of the record gives a measure of the relative effect of the drugs on the tonus of the intestine. Parpanit in amounts of 0.5 mgm/kgm and 0.25 mgm/kgm. caused average decreases in the level of the record of 4.54 cm in 16 experiments and 3.79 cm in 6 experiments, respectively. Trasentine with 2.0 mgm/kgm and 1.0 mgm/kgm caused average decreases of 3.75 cm in 8 experiments and 3.14 cm in 7 experiments, respectively. Atropine in 14 experiments with 0.05 mgm/kgm produced an average drop of 5.15 cm. On the basis of these observations, atropine appears to be over 10 times as active as Parpanit in relaxing the dog intestine and Parpanit approximately 8 times as active as trasentine.

The height of the spontaneous contractions was reduced by all 3 drugs. Parpanit in a dosage of 0.25 mgm/kgm reduced the contractions in 3 of the 6 experiments and 0.5 mgm/kgm in 10 of the 15 experiments (see figure 3). Trasentine in a dosage of 1 mgm/kgm reduced the contractions in 1 of the 7 experiments and 2 mgm/kgm in 6 of the 10 experiments. Atropine was most powerful, inhibiting the rhythmical contractions in 11 of the 14 experiments.

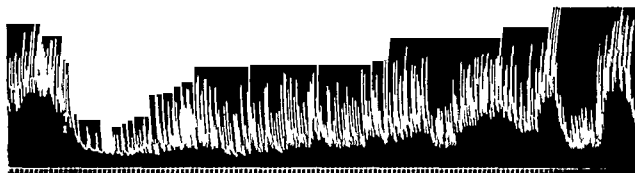


FIG 3 Dog ♀ 9.2 kgm. Thirty Vella loop. Upper curve is a record of activity of the loop of gut with rubber balloon at a pressure of 20 cm. of water, lower line time in intervals of 20 seconds. 1. Parpanit 0.5 mgm/kgm was injected intravenously.

Atropine in this effect is apparently over 10 times as active as Parpanit and Parpanit over 4 times as active as trasentine.

The duration of action of these drugs was of the same order as their relative potency. Parpanit in a dosage of 0.5 mgm/kgm generally relaxed the intestine for a period of from 15 to more than 30 minutes, while 0.25 mgm/kgm was generally effective for 15 minutes or less. Trasentine in a dosage of 2 mgm/kgm depressed the intestine for from 3 to 15 minutes, with one experiment showing a longer depression. A dose of 1 mgm/kgm of trasentine usually depressed intestinal tone for only 2 to 3 minutes, with one experiment slightly longer. The effect of atropine was prolonged, only one animal exhibiting a return of intestinal tone during a period of 20 to 40 minutes following the injection.

*Isolated Intestine* Segments of intestine in pairs, one piece each from duodenum or jejunum and ileum, were suspended longitudinally in a bath of Tyrode's solution kept at 38.5°C (8). To this 100 cc bath were added aqueous solutions of the drugs in amounts sufficient to make the desired final concentrations. A total of 26 pairs of segments from 15 rabbits was used in this series.

Two types of experiments were carried out with this preparation. 1) the effects

of Parpanit, trasentine and atropine individually on the activity of the segments and, 2) a comparison of their ability to antagonize the action of acetylcholine.

1. In the first series, 50 experiments were performed with Parpanit, 38 with trasentine and 30 with atropine. The principal effect of all 3 drugs was to cause a decrease in the height of the contractions of the segment. Table 3 reveals that Parpanit in a concentration of 1:1,000,000 was depressing to contractions in 14 of the 20 pairs of segments and that Parpanit and trasentine are essentially equal in depressing the isolated rabbit intestine, while atropine sulfate is somewhat less depressing. The rate of the spontaneous contractions was not markedly reduced until the concentration of either Parpanit or trasentine reached 1:200,000 while atropine in the concentrations studied did not affect the rate.

2. In studying the antagonism of these drugs to acetylcholine, the acetylcholine was added to the bath in different experiments either 3 minutes before, simultaneously with, or 3 minutes after the addition of the drug under study. Within this range, the results remained constant. All of the drugs including

TABLE 3  
*Effect on amplitude of contractions in isolated rabbit intestine*

CONCENTRATION	PARPANIT		TRASENTINE		ATROPINE	
	A	B	A	B	A	B
1 1,000,000	20	70				
1 500,000	28	71 4	14	78 6	12	41 7
1 200,000	20	85	30	83.3	24	54.2
1 100,000	28	85 7	22	72.7	19	31 6

A—Number of experiments

B—Per cent of experiments in which inhibition was observed.

acetylcholine were used in concentrations of from 1:100,000 to 1:10,000,000, 57 experiments being performed with Parpanit, 48 with trasentine and 37 with atropine sulfate.

Parpanit was found to antagonize the stimulating action of acetylcholine on the intestine segments, the degree of inhibition depending on the relative concentrations of the 2 drugs. All experiments in which the gravimetric ratio of Parpanit to acetylcholine was 1:1 or higher showed some inhibition of acetylcholine action. Atropine was well over 10 times as potent and trasentine markedly less active, having perhaps one-tenth the potency of Parpanit.

Records from comparative experiments are shown in figure 4. Parpanit, trasentine and atropine in concentrations of 1:300,000 were followed in 3 minutes in their respective experiments by acetylcholine to a concentration of 1:500,000. The antagonism by atropine was nearly absolute, that by Parpanit less but marked and that by trasentine least of all. The effect of the atropine was far more persistent than that of either of the other 2 drugs.

*Intact Uterus.* Contractions of the uterine horns of 8 rabbits under urethane anesthesia and 6 decerebrated cats were recorded by a method described by one of us (9).

Parpanit administered intravenously to the rabbits in doses of from 2 to 5 mgm /kgm resulted in stimulation of uterine contractions in 10 of the 23 experiments and no action in 13. Trasentine in equal amounts resulted in stimulation in 6 of the 13 and was without effect in 7. These results are at variance with those of Johnson and Reynolds (10) who found trasentine in intravenous doses of 2 to 4 mgm /kgm to be without any effect on the rhythmic contractions of the rabbit uterus. They were, however, recording from uterine fistulae in unanesthetized animals, whereas, in the experiments described here the rabbits were urethranized and the abdomens were open. Intravenous injections of 2 to 4 units of posterior pituitary solution produced marked stimulation in all animals, as did 1 100,000 epinephrine solution in amounts from 0.2 to 1.0 cc

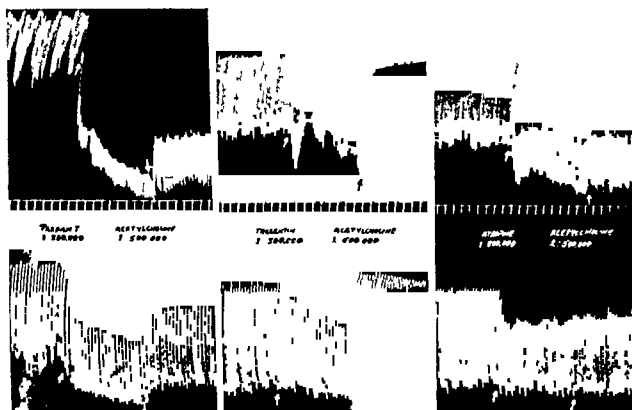


FIG 4. Excised longitudinal segments of rabbit intestine. In each case the upper curve is that of the duodenum and the lower curve that of the ileum, time in intervals of 20 seconds.

In the cats, 2 to 5 mgm /kgm of Parpanit evoked stimulation of contractions in 11 of the 13 experiments and similar amounts of trasentine caused stimulation in 5 experiments. Figure 5 is a typical record of these results in which the drugs caused increased activity of the uterus. Injections 1 and 5 are omitted from the record to conserve space. The first injection which was trasentine was the same as that seen at 3 in the record and the other injection omitted was that of epinephrine (0.5 cc of a 1 100,000 solution) which caused a decrease in both the general tonus and force of the rhythmical contractions of the uterus. As can be seen at 2, 3, and 4, Parpanit, trasentine and posterior pituitary solution, respectively, caused a sudden and pronounced increase in the general tonus of the intact cat uterus. All of the intravenous injections of posterior pituitary solution induced strong contractions and all injections of epinephrine solution caused marked relaxation.

*Excised Uterus.* Pairs of segments of non-pregnant uteri, one from each horn, were suspended in aerated Locke's solution adjusted to pH 7.6 and kept at 38°C. for recording their activity (11). Twelve pairs of segments from rabbit.

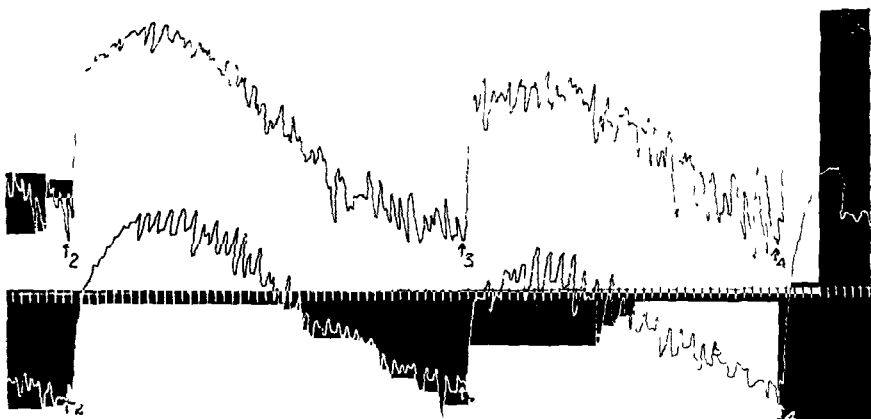


FIG. 5. Decerebrated cat ♀ 2.2 kgm. Records taken from two uterine horns. Upstroke, contraction, downstroke, relaxation. Time in intervals of 20 seconds. 2. Parpanit 3 mgm./kgm. injected intravenously; 3. Trasentine 3 mgm./kgm.; 4. Surgical pituitrin, 6 units.

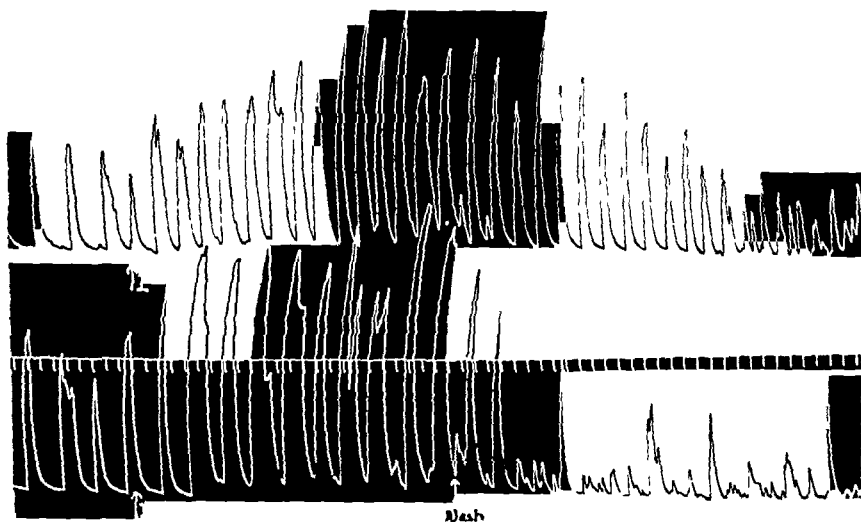


FIG. 6. Excised rabbit uterine segments in Locke's solution. Time in intervals of 20 seconds. 1. Parpanit 1:200,000 W—wash.

uteri, 7 pairs from rats and 4 from cats were used, with results from Parpanit and trasentine constant for all animals.

Stimulation of the uterine segments, regardless of the control state of activity, was regularly obtained by Parpanit in a concentration of 1:100,000 in the bath.

Fifty experiments with this concentration of Parpanit resulted in stimulation in 48. At a level of 1:1,000,000, Parpanit was stimulating in 8 of the 15 experiments and without effect in 7. Figure 6 illustrates the stimulation of active segments of rabbit uterus by Parpanit in a concentration of 1:200,000. Inhibition of contractions was observed with concentrations ranging in various experiments from 1:20,000 to 1:5,000, the decrease in contraction height sometimes being accompanied by an increase in tone. Uteri inhibited by Parpanit at these levels were refractory to stimulation by posterior pituitary solution or epinephrine (rabbit). The contractions stimulated by pituitrin were never antagonized by Parpanit except in the high concentrations necessary to depress the uterus in the absence of pituitrin, probably a toxic manifestation. On the contrary, pituitrin-induced contractions, if not maximal, were augmented by

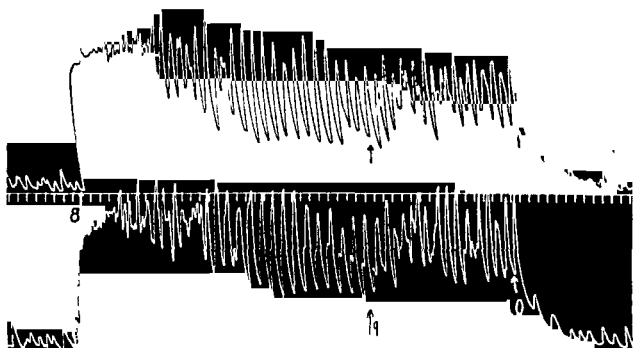


FIG. 7. Excised rabbit uterine segments. Locke's solution. Time in intervals of 20 seconds. 8. Posterior pituitary solution 0.05 unit in 100 cc. bath. 9. Parpanit 1:100,000. 10. Wash

stimulating concentrations of Parpanit. This augmenting effect of Parpanit on pituitrin-stimulated contractions is shown in figure 7.

Parpanit in stimulating concentrations tended to produce increased height of contractions, increased rate of contraction and increased tonus, in that order. In 20 experiments with segments actively contracting, Parpanit in a concentration of 1:100,000 caused an increased amplitude in 19, increased rate of contraction in 16 and increased tone in 10. In 26 experiments with quiescent segments, a similar concentration of Parpanit initiated contractions in 24 and increased the tonus in 4.

Trasentine appeared essentially similar though possibly not quite as active in its effects on the uterus. Using a concentration of 1:100,000, stimulation was observed in 23 of the 28 experiments. Lower concentrations were less active, while depression of contractions occurred in concentrations of from 1:20,000 to



1:10,000. Trasentine, like Parpanit, was found to antagonize pituitrin only in toxic concentrations and in lower concentrations to augment its action. This is in marked contrast to the observations of Johnson and Reynolds (10) to the effect that the activity of the *intact* rabbit uterus stimulated by pituitrin is significantly reduced by trasentine in doses of 3 mgm./kgm.

In 14 experiments with atropine sulfate in concentrations of from 1:100,000 to 1:1,000,000, mostly on rat uteri, no effect was observed.

Posterior pituitary solution in amounts of from 0.01 to 0.05 units in the 100 cc. bath and epinephrine solution in concentrations of 1:10,000,000 to 1:100,000,000 were used in 35 and 11 experiments, respectively, with all segments showing the usual responses.

#### SUMMARY AND CONCLUSIONS

1. The LD<sub>50</sub> of diethylaminoethyl ester of phenyl-cyclopentane-carboxylic acid as the hydrochloride, "Parpanit", is: 24.5 mgm./kgm. intravenously for rabbits; 45.1 mgm./kgm. intravenously for mice; 222.3 mgm./kgm. intraperitoneally for mice; and 209.0 mgm./kgm. intraperitoneally for rats.

2. Toxic doses of Parpanit produce symptoms of central nervous system stimulation in rabbits, rats and mice, with death ultimately due to respiratory paralysis.

3. There is no evidence in rats of the development of tolerance to large amounts of the drug.

4. Moderate amounts administered to dogs produce tachycardia by peripheral inhibition of the vagus, a brief hypotension from vasodilatation by local action, and a temporary depression of respiration.

5. In addition to inhibiting the effect on the dog heart of the artificially stimulated vagus, Parpanit inhibits the effect of injected acetylcholine on the heart rate, but not on the blood pressure.

6. The inhibition of the turtle heart by the vagus is decreased by Parpanit in dilute solution.

7. Parpanit depresses the contractions of the perfused frog ventricle in dilute solution.

8. Parpanit antagonizes the action of acetylcholine on the perfused isolated guinea pig lung and to a lesser degree the action of histamine.

9. Spontaneous activity and tone are both diminished in the intact intestine of the dog by Parpanit.

10. Dilute solutions of Parpanit depress the activity of isolated strips of rabbit small intestine, especially the amplitude of the contractions.

11. Similar solutions depress the responses of isolated gut segments to acetylcholine.

12. In many instances the uterus of the decerebrated cat and the urethanized rabbit is stimulated by Parpanit. No instances of relaxation were observed.

13. In contrast to the isolated intestine, isolated uterine segments of rabbit, cat and rat are strongly stimulated by dilute solutions of Parpanit, depression occurring only when strong solutions are used. The action of pituitrin is aug-

mented by Parpanit in dilute solutions and antagonized only by toxic concentrations.

14. Parpanit and trasentine are qualitatively similar but have quantitative differences in different types of experiments. They are approximately equal in potency in depressing the isolated intestine, in inhibiting the action of acetylcholine on the guinea pig lung, and in stimulating both intact and isolated uteri. Parpanit is more active in inhibiting the action of acetylcholine on the isolated intestine, in relaxing the intact intestine, and in depressing the turtle cardiac vagus nerve, and is a trifle more depressing to the frog ventricle.

15. Atropine is far more active in inhibiting the vagus nerve and in blocking the actions of acetylcholine and is less depressing to the isolated intestine and the frog ventricle. In the concentrations used it is without effect on the uterus.

#### REFERENCES

1. DOMENJOZ, R.: Schw. Med. Wochen., 76: 1282, 1946.
2. HEYMANS, C., AND DE VLEESCHOUWER, G. R.: Arch. Internat. Pharmacodyn. et de Therap., 75: 307, 1948.
3. CHARLIER, R., AND PHILIPPOT, E.: 1948, Personal communication.
4. LEHNER, F., AND LANINI, G.: Helv. Physiol. Acta, 6: 356, 1948.
5. SOLLMANN, T., AND VON OETTINGEN, W. F.: Proc. Soc. Exper. Biol. and Med., 25: 692, 1928.
6. TANTER, M. L., PEDDEN, J. R., AND JAMES, M.: THIS JOURNAL, 51: 371, 1934.
7. GRUBER, C. M., HART, E. R., AND GRUBER, C. M., JR.: THIS JOURNAL, 73: 319, 1941.
8. GRUBER, C. M., AND GRUBER, C. M., JR.: Arch. Internat. Pharmacodyn. et de Therap., 63: 244, 1939.
9. HALBEISEN, W. A., GRUBER, C. M., JR., AND GRUBER, C. M.: Proc. Soc. Exper. Biol. and Med., 68: 343, 1948.
10. JOHNSON, J. R., AND REYNOLDS, S. R. M.: THIS JOURNAL, 59: 365, 1937.
11. GRUBER, C. M., SCHOLTEN, R., DENOTE, A., AND WILSON, J. F.: THIS JOURNAL, 56: 341, 1936.

# PLASMA NEOSTIGMINE LEVELS AND CHOLINESTERASE INHIBITION IN DOGS AND MYASTHENIC PATIENTS<sup>1</sup>

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This is an investigation into the interrelations of dose, route of administration, excretion, destruction and plasma level of Neostigmine, U.S.P. XIII (Prostigmine) in dogs and in patients with myasthenia gravis. Few quantitative studies of this kind have been reported and these (1, 2, 3) do not present a consistent picture. Previous work from this laboratory (4, 5) showed that gross errors are inherent in the usual techniques for determination of *in vivo* cholinesterase activity in the presence of reversible inhibitors. Full details are to be found in this earlier work, especially in the study of physostigmine in dogs (6), to which the present report is in large part a sequel.

The following principles were demonstrated with physostigmine: (a) During the period of determination of the plasma cholinesterase activity physostigmine is competitively displaced by acetylcholine. This *competition effect* must be corrected for. (b) When eserinated serum is diluted (usually tenfold) prior to the manometric determination, the enzyme-inhibitor complex dissociates reversibly so that the degree of inhibition is markedly reduced. This *dilution effect*, which should occur with any reversible system, must be controlled with respect to time, and corrected for. (c) Enzymatic destruction of physostigmine may occur before the determination is completed.

Since Prostigmine levels were to be calculated from the degree of inhibition of plasma cholinesterase, a thorough understanding of the isolated Prostigmine-cholinesterase system was first required. Thus the first section of this paper consists of *in vitro* experiments, performed with a view to the proper correction of animal or human data to *in vivo* conditions. The second and third sections comprise the work with dogs and myasthenic patients, a preliminary report of which has already appeared (7).

## I. Action of Prostigmine on cholinesterase activity of dog serum *in vitro*

*Method.* The manometric method of Ammon (8) is used throughout. Dog serum, appropriately diluted with bicarbonate-Ringer solution containing Prostigmine methylsulfate is allowed to stand overnight at 0°C. to allow full combination with the enzyme (9). Then 2.0 cc. of the enzyme solution are placed in the main Warburg vessel and 0.2 cc. of 20 per cent acetylcholine bromide (recrystallized) in the side-bulb. The acetylcholine concentration after mixing is always 0.0805 molar. Equilibration for 7 minutes at 37° with 95 per cent N<sub>2</sub>-5 per cent CO<sub>2</sub> is followed by tilting 5 minutes later and the initial reading is

<sup>1</sup> These studies were supported by grants from Hoffman-LaRoche, Inc., the Ella Sachs Plotz Foundation, and the William W. Wellington Memorial Research Fund.

taken 3 minutes after the reaction has started. The usual period of measurement is then 20 minutes, although in some of the experiments successive 20-minute periods are considered.

*The inhibition curve.* Figure 1 shows the results of a number of experiments in which the 20-minute reaction rates are plotted as *fraction of normal activity (a)* against the logarithm of the molar Prostigmine concentration. The fractional activity for a given inhibitor concentration is predictable with a standard error of approximately  $\pm 0.034^2$ .

The shape of the curve and its mid-point slope (about 0.575) are the same as for physostigmine and indicate a combination of one molecule Prostigmine with

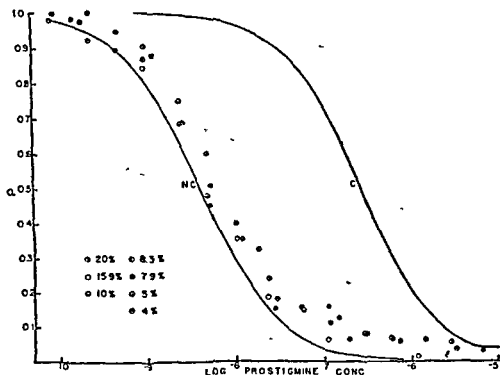


FIG. 1. INHIBITION OF DOG SERUM CHOLINESTERASE BY PROSTIGMINE

...ive curve in the cholinesterase,

a single enzyme active center (4). This slope is to be expected whenever an enzyme-inhibitor system operates in zone A, where  $E'$  is less than  $0.1^2$ , a negligible fraction of total inhibitor being combined with enzyme even at full inhibition. Under these conditions the inhibition should not depend upon the enzyme concentration and figure 1 shows that this is true, at least over the range 4 per cent to 20 per cent serum. The degree of saturation of an enzyme by its inhibitor may be thought of as depending on the "thermodynamic pressure" of free inhibitor. To maintain half the enzyme centers in a combined, inactive

<sup>2</sup> In the absence of a theoretical linear curve, approximate standard errors were calculated here (and also in figures 6 and 17) from the expression  $\pm \sqrt{\frac{\sum d^2}{n-2}}$ , deviations being taken from the points to an empirical curve of best fit.

<sup>3</sup>  $E'$  is defined as  $E/K$ —the concentration of enzyme active centers divided by the dissociation constant of the enzyme-inhibitor complex.

state, for example, requires the same concentration of *free* inhibitor molecules whether there are many or few enzyme centers present.

The position of the curve on the axis of abscissae shows that the affinity of Prostigmine for the enzyme is considerably greater than that of physostigmine, the activity being reduced 50 per cent by a concentration less than  $10^{-8}$  molar. Exact comparison of affinities requires calculation of the Prostigmine-enzyme dissociation constant and this cannot be done from the observed points of figure 1. These 20-minute values represent transitional stages in the increase of activity resulting from displacement of inhibitor by substrate. To obtain a true picture of the inhibition in each case before addition of acetylcholine one must consider the competition effect in some detail.

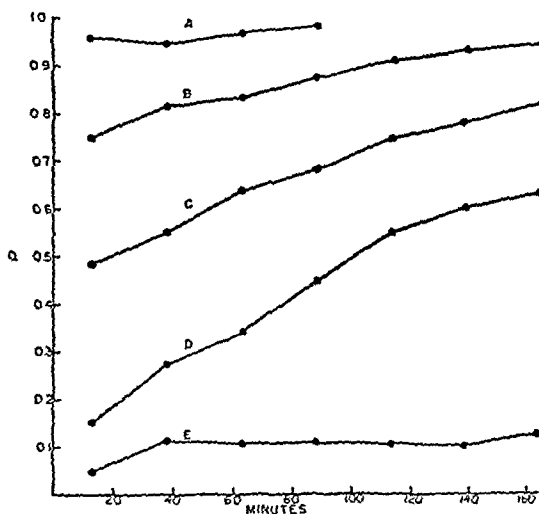


FIG. 2. COMPETITIVE DISPLACEMENT OF PROSTIGMINE BY ACETYLCHOLINE

Prostigmine incubated overnight with 10% dog serum. Acetylcholine added at zero time. Successive 20-minute activities plotted at mid-point of each interval. A:  $4.50 \times 10^{-10}$  M/L. B:  $2.22 \times 10^{-9}$ . C:  $4.44 \times 10^{-9}$ . D:  $1.11 \times 10^{-7}$ . E:  $1.39 \times 10^{-5}$ .

*Competition.* In figure 2 are plotted the results of five experiments at different Prostigmine concentrations, in which the reactions, after addition of substrate, were allowed to run for seven successive 20-minute periods. The activities are expressed as fraction of normal and plotted at the midpoints of each time interval.<sup>4</sup>

The curves show a gradual displacement of inhibitor by substrate. Two states are therefore to be distinguished—an initial non-competitive equilibrium between inhibitor and enzyme, and a final competitive equilibrium in which Prostigmine, acetylcholine and cholinesterase all participate. The inhibition

<sup>4</sup> The normal rate falls off slowly as the reaction proceeds, an effect which is accurately predictable on the basis of the total  $\text{CO}_2$  liberated from the beginning of the reaction. Consequently, inhibited rates in continuous runs are compared with the normal at the same total  $\text{CO}_2$  evolution.

curves representing these two equilibria have been arrived at on the theoretical basis outlined elsewhere (5) and appear as NC and C in figure 1. The effect of the separation of these two curves is that at equilibrium nearly 100 times as much Prostigmine is required to produce a given inhibition in the presence of this concentration of acetylcholine as in its absence. The Prostigmine-cholinesterase dissociation constant, obtained from curve NC (when  $a = 0.5$ ), is  $3.89 \times 10^{-9}$ , as compared with  $3.11 \times 10^{-8}$  for physostigmine.

The kinetics of competition have been treated in some detail for physostigmine (5). It was shown that the initial rate of change of  $a$  can be described by the equation

$$\left(\frac{da}{dt}\right)_{a=0} = \frac{S'}{k_1} + \frac{S'}{k_2}$$

Here  $S'$  is the acetylcholine concentration expressed in units of its enzyme-complex dissociation constant ( $S/K$ ) and  $k_1$  is the rate constant of dissociation of this complex, and  $I'$  and  $k_2$  have similar meanings with respect to the inhibitor. For all comparable inhibitors of this enzyme a particular value of  $I'$  produces the same fractional activity. It follows that as between several inhibitors the only variable in the rate equation is the constant  $k_2$ ; that this constant alone determines the rate of increase in activity from any initial value of  $a$ ; and consequently that *if the rates of displacement are of comparable magnitude, so must be the several rate constants,  $k_2$ .*

When we compare the curves of figure 2 with analogous curves for physostigmine (5, p. 565), we find that the competition rates are essentially the same. We may therefore conclude that  $k_2$ , the rate constant of dissociation is of the same order for both compounds. The dissociation constant,  $K$ , is the ratio  $k_2/k_1$ , where  $k_1$  is the rate constant of association. But  $K$  (Prostigmine) is about one-eighth as great as  $K$  (physostigmine). It follows that  $k_1$  must be about *eight times as great*. In other words, once the complex is formed its *rate of dissociation is about the same* for the two inhibitors. The greater affinity of Prostigmine must be explained by its greater rate of combination at equimolar concentration. Since the two inhibitors do not differ greatly in molecular size the effect is presumably a result of a higher proportion of effective collisions on the part of Prostigmine.

**Dilution.** The theory of the dilution effect has been discussed elsewhere (6, p. 21). A curve of best fit to the observed 20-minute points of figure 1 was corrected for competition and dilution, to yield the correction curve of figure 3. The ordinates represent fractional activity of undiluted sera while the abscissae show the fractional activity observed in the same sera after tenfold dilution. For example, an actual activity of 0.42 would give an apparent activity, after dilution, of 0.90.

A curious distortion is evident. The whole range 0.42 to 1.0 before dilution is compressed into a small range of observed values between 0.90 and 1.0. On the other hand, actual activities below 0.05 before dilution are spread out between observed values 0 and 0.45. As the reliability of any single determination of fractional activity ( $\pm 3$  per cent) applies to observed values after dilution it becomes impossible to estimate with much accuracy the activities of undiluted sera unless these activities are very low. Differences between such low activities (eg. 0.03-0.04) can readily be detected. *In the subsequent sections cor-*

rected fractional activities will always appear in italics, uncorrected activities in roman type.

**Destruction.** The enzymatic destruction of Prostigmine by dog serum at 37° was studied. Under the conditions of these experiments non-enzymatic breakdown is negligible. Previous work (5) had demonstrated that the enzymatic destruction of physostigmine could be considered as a special case of the destruction of any compound acting as a *substrate*, despite the extremely low hydrolysis rate. This rate depends upon the degree of saturation of the enzyme and upon a rate constant of destruction ( $k_D$ ) known as the "turnover number", which is characteristic of each substrate. Experiments with highly purified plasma cholinesterase indicate that acetylcholine, Prostigmine and physostig-

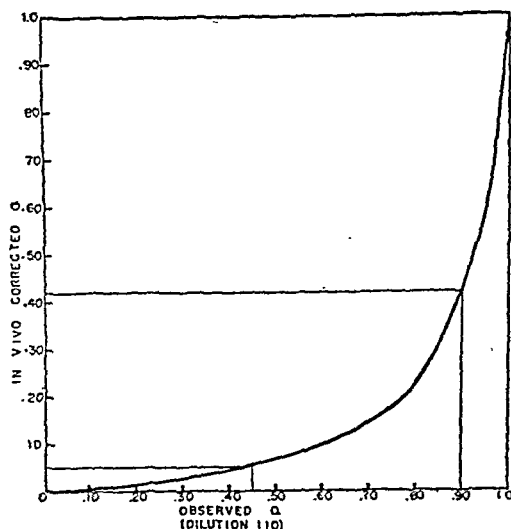


FIG. 3. EFFECT OF DILUTION UPON THE ACTIVITY OF DOG SERUM CHOLINESTERASE INHIBITED BY PROSTIGMINE

mine are hydrolyzed by the same enzyme (11). This is indicated also by the course of Prostigmine destruction described here. The general equation for destruction in such a system was shown to be

$$\left[ \frac{1}{a_0} + \ln \frac{1 - a_0}{a_0} \right] - \left[ \frac{1}{a} + \ln \frac{1 - a}{a} \right] = k_D E' t$$

where  $a_0$  is the initial fractional activity,  $E' = E/K$  as defined on p. 563(4), and  $a$  is the fractional activity at any time,  $t$ .

The general course of inhibitor breakdown, expressed in terms of  $a$  as a function of  $k_D E' t$  is shown in figure 4. At low values of  $a$ , while the rate of destruction of Prostigmine is maximal, the change in  $a$  is very slow. The steepest slope of the curve and most rapid increase in activity occurs when  $a = 0.667$ . Consequently, as far as enzymatic destruction alone is concerned, low activities

(below 0.20) should persist while moderate activities should increase rapidly toward normal.

For reasons explained elsewhere (4), a definitive value of  $E'$  cannot be obtained, at least in this system. However  $k_D E'$  can be handled conveniently as a unit. While  $k_D$  will have a fixed value in the case Prostigmine-cholinesterase,  $E'$  will vary in proportion to the serum concentration. Furthermore it will depend directly upon the concentration of enzyme active centers, which is proportional to the activity of uninhibited normal serum with acetylcholine as substrate.

In order to determine the constants in the destruction equation, Prostigmine was incubated at  $37^\circ$  with 22.7 per cent dog serum. The normal enzyme activity of this solution was 0.392 mM acetylcholine hydrolyzed per liter per minute. After overnight equilibration an initial determination (in duplicate) gave  $a_0 = 0.38$ . Twenty-four hours later,  $a$  was

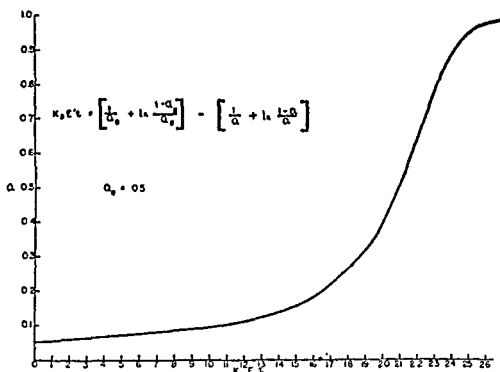


FIG. 4. THEORETICAL COURSE OF RECOVERY OF CHOLINESTERASE ACTIVITY DURING ENZYMATIC DESTRUCTION OF AN INHIBITOR

For explanation, see text

0.77 and after 48 hours 0.98. If these values of  $a$  are located on the curve of figure 4 it will be found that a difference of 3.1 and 6.2 units, respectively, is represented by their abscissal projections. Dividing  $k_D E' t$  by  $t$  (minutes) gives the same result for both sets of determinations, namely,  $k_D E' = 2.15 \times 10^{-3}$  in this serum solution.

The value of  $k_D E'$  at other concentrations and in other sera should depend upon the normal enzyme activity; it should be equal to  $2.15 \times 10^{-3} \times \text{normal activity}/0.392$ , where normal activity is always expressed as mM/L/min.

To check on the validity of this expression in predicting destruction rates we incubated Prostigmine as above, but substituted a 9.1 per cent serum solution having the low normal enzyme activity 0.0782 mM/L/min. The initial fractional activity in the presence of Prostigmine was 0.43 which increased after 49 hours to 0.57. From the formula above  $k_D E' = 4.3 \times 10^{-4}$  and multiplying by  $t$  we obtain  $k_D E' t = 1.3$ . On figure 4, the abscissal value for  $a_0 = 0.43$  is 20.3; increasing this by 1.3 gives 21.6 and an ordinal value  $a = 0.58$ . In other words, the predicted activity after 49 hours was 0.58 and the experimentally determined value was 0.57.



It is of some interest to compare the destruction rates of Prostigmine, physostigmine and acetylcholine. If the term  $k_D E'$  be divided by the dissociation constant for each substance, three values are obtained for the term  $k_D E$ . But  $E$ , which is the molar concentration of enzyme centers, will be the same in all three cases. The turnover numbers,  $k_D$ , can then be compared. It has been shown (4) that the slope of the inhibition curve (figure 1) would be significantly steeper if  $E'$  were greater than unity at 20 per cent serum concentration. This fact allows one to assign a *maximal* value,  $E < 2 \times 10^{-8}$  M in whole serum, a figure one-twentieth that previously assigned as a result of the work with physostigmine. Dividing each  $k_D E$  by this maximal  $E$  gives *minimal* estimates of the turnover numbers. These data are summarized in table 1.

The portions of the foregoing discussion that bear upon determination of the *activity of inhibited cholinesterase in vivo* may be summarized as follows: Serum containing Prostigmine is withdrawn from an animal and diluted. The frac-

TABLE 1

*Dissociation constants and turnover numbers for acetylcholine, physostigmine and Prostigmine*

SUBSTRATE	$k_D E'$	K	$k_D E$	$k_D$ (PER MINUTE)
Acetylcholine (5)	1.48	$1.25 \times 10^{-3}$	$1.85 \times 10^{-3}$	>100000*
Physostigmine (5, 10) (horse serum)	1.4	$3.11 \times 10^{-3}$	$4.2 \times 10^{-8}$	>2.1
Prostigmine†	0.009	$3.89 \times 10^{-9}$	$3.5 \times 10^{-11}$	>0.0018

\* Cf. 89400 per minute for horse serum cholinesterase (8).

† The average normal activity of all the sera used was 1.63. The value of  $k_D E'$  given here is from the expression  $2.15 \times 10^{-3} \times 1.63/0.392$ .

tional activity of this serum is determined by measuring the amount of acetylcholine hydrolyzed in a 2½-minute period following its addition. The value of  $a$  thus arrived at is somewhat too high as a result of competition and very much too high as a result of dilution. The destruction of Prostigmine in 20 minutes is negligible. By applying the corrections depicted in figure 3 the fractional activity obtaining *in vivo* can be found but the accuracy of such data varies with the degree of inhibition. *Prostigmine concentrations* can be found directly from the observed fractional activity and the uncorrected points of figure 1. The values thus obtained are simply multiplied by the dilution factor to give Prostigmine concentrations *in vivo*.

## II. Prostigmine levels and cholinesterase inhibition in dogs

*Method.* Dogs weighing from 15 to 25 kgm. were given Prostigmine methylsulfate by mouth, by single intravenous injection, or by continuous intravenous infusion. The continuous infusions were conducted in 26 dogs, as described elsewhere (6) (q.v. for typical protocols), except that no single animal was ever used for more than two infusion rates. The dogs were anesthetized, Pentobarbital Sodium<sup>8</sup> (0.035 gm. per kgm.) being used intraperitoneally to initiate anesthesia, which was then maintained by administration of the

<sup>8</sup> Nembutal, kindly supplied by Abbott Laboratories, North Chicago, Illinois.

same drug (0.075 per cent in physiological saline) into the femoral vein at a rate between 0.5 and 2.5 cc. per minute. Body temperature was recorded and maintained near normal. Respiration was spontaneous. Respiratory and heart rates were recorded throughout each experiment and blood pressure at the termination of the experiment. That the animals remained in good condition is indicated by the fact that the terminal blood pressures were above 110 mm. except in one experiment where it was 80 mm. after 13 hours anesthesia and two experiments where the kidneys had been ligated and the animals died after 30 hours. Hematocrit determinations were periodically performed and saline administered to prevent hemoconcentration.

Blood samples of 6 cc. each were taken from the right external jugular vein. The blood was allowed to clot, was centrifuged, and the serum removed, diluted and stored at 5° for at least nine hours. Storage of the diluted serum before determination is to insure completion of the dissociation which takes place on dilution. Dilutions were 1 to 10 unless otherwise specified; the diluent was the bicarbonate-Ringer solution used for the determinations. The normal cholinesterase activity was established by averaging three blood samples taken prior to Prostigmine administration. These normal activities varied between 0.926 and 3.10 mM/L/min. with an average of 1.63. There is no significant change in the serum activity of the normal dog during 36 hours, the maximum duration of an experiment.

Except at very low infusion rates, atropine sulfate (1 mgm. per 10 mgm. Prostigmine methylsulfate) was included in the saline Prostigmine solution which was infused into the femoral vein at a constant rate of 5 to 10 cc. per hour by means of a calibrated automatic infusion pump. Additional amounts of atropine sulfate (0.5 to 1.0 mgm.) were given when needed to counteract severe toxic effects, not only in the continuous infusions but in the experiments with single oral or intravenous doses as well. Neither atropine sulfate, in the amounts used, nor pentobarbital anesthesia have any effect upon the normal cholinesterase activity.

*Continuous infusion.* 1. *Plasma Prostigmine levels.* Figure 5 shows that constant levels of plasma cholinesterase inhibition can be established by continuous infusion of Prostigmine at a constant rate. The curves represent the observed values in nine representative experiments. The corrections have not been applied and the points therefore do not represent actual enzyme inhibition in the circulating plasma.

The fixed relationship of enzyme inhibition to Prostigmine concentration has already been shown *in vitro*. The constant inhibition levels attained here therefore represent constant Prostigmine levels in the plasma. Disposal mechanisms are evidently flexible enough to keep pace with a wide range of infusion rates ( $0.134 - 1040 \times 10^{-10}$  M/kgm./min.) or steady states could not be established.

The higher the infusion rate the more rapidly a steady state level is achieved. As the rate of infusion is progressively increased the change in steady state inhibition becomes smaller and smaller. It is ever more difficult to depress the cholinesterase activity as lower and lower values are reached and more and more enormous amounts of Prostigmine are infused. To some extent this is merely a result of the sigmoid shape of the semi-logarithmic inhibition curve (figure 1), ever larger inhibitor concentrations being required to produce small increments of inhibition as complete inhibition is approached. But quantitatively the effect is far greater than could be accounted for on this basis alone.

In figure 6 are plotted the steady state levels finally achieved in 23 experiments at various infusion rates. Each steady state activity is plotted against the logarithm of the infusion rate, expressed in moles per kilogram per minute.

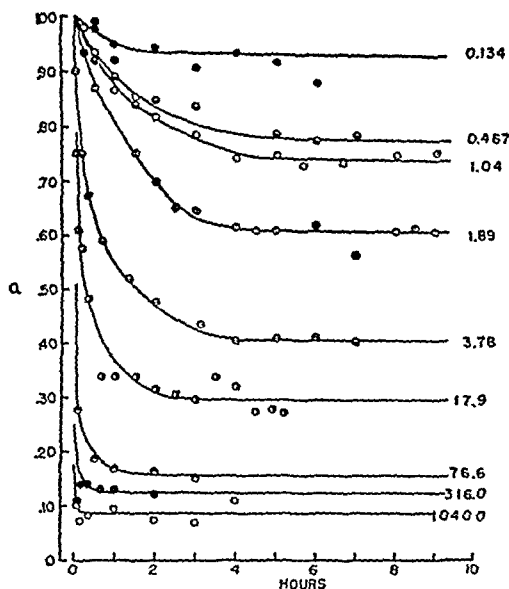


FIG. 5. ESTABLISHMENT OF STEADY STATE CHOLINESTERASE ACTIVITIES BY CONTINUOUS INTRAVENOUS INFUSION OF PROSTIGMINE IN DOGS

The values of  $a$  are uncorrected. Figures to the right of each curve refer to infusion rates in moles  $\times 10^{-10}$  per kilogram per minute.

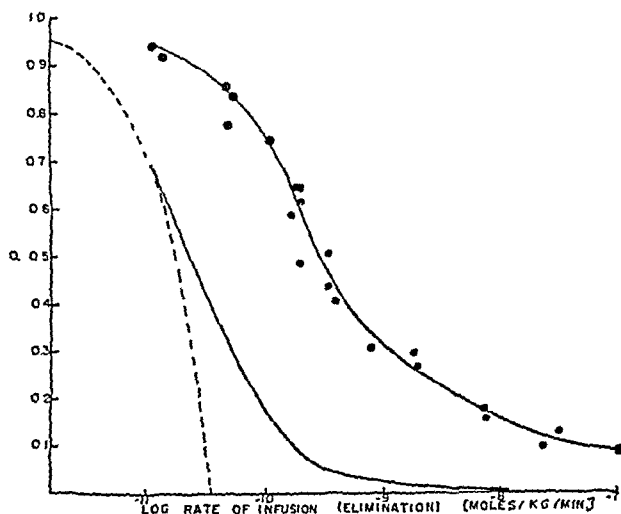


FIG. 6. RELATIONSHIP OF STEADY STATE CHOLINESTERASE ACTIVITY TO RATE OF PROSTIGMINE INFUSION IN DOGS

The values of  $a$  are uncorrected. There are two points, nearly superimposed, at the extreme right of the curve. The curve of best fit is shown corrected to *in vivo* conditions in the lower solid curve. The broken line represents destruction of Prostigmine by plasma.

At a given infusion rate the uncorrected steady state activity can be predicted with a standard error of approximately  $\pm 0.039$ , a reliability comparable with that of *in vitro* determinations of inhibited enzyme activity (cf. footnote 2).

The entire curve drawn through the observed points has been corrected so that the lower solid curve represents the true activities *in vivo*.

2. *Elimination mechanisms.* When a steady state has been reached, the rate of infusion must exactly compensate for all disposal mechanisms. Thus *at the steady state, infusion rate is equal to elimination rate*. The axis of abscissae in figure 6 is therefore labelled *elimination* as well as *infusion*.

a. *Destruction by plasma.* Overall elimination is the result of an unknown number of separate mechanisms. The most obvious of these, the enzymatic destruction of Prostigmine, has already been analyzed. From the equation  $-(dI/dt) = k_D(EI) = K \cdot k_D E' (1-a)$  and the value of  $k_D E'$  obtained earlier, the rates of destruction of Prostigmine at the various values of  $a$  have been plotted as the broken curve of figure 6. It will be observed that enzymatic destruction accounts for a significant portion of the total elimination only at high fractional activities. At low values of  $a$  the total elimination becomes enormously greater than the enzymatic destruction by plasma, which cannot exceed a maximal rate (when the plasma cholinesterase is saturated) of  $3.5 \times 10^{-11}$  moles per liter per minute.<sup>6</sup>

When the ordinates of figure 6 are converted to Prostigmine concentrations with the aid of figure 1 the relation between plasma drug level and elimination rate is obtained. This is presented graphically in figure 7. The broken curve again represents the calculated rate of enzymatic destruction in plasma. In contrast to the limiting rate attained by this curve, the points representing overall elimination show no tendency to reach a maximum. The rate of elimination increases with the Prostigmine concentration up to the highest level examined.

b. *Renal excretion.* What are the mechanisms responsible for Prostigmine elimination at concentrations higher than that required to saturate the plasma enzyme? If the kidneys are ligated after the establishment of a steady state, and the infusion continued at the same rate, the steady state should be upset if the kidneys play an important role in elimination. In figure 8 are plotted the results of four experiments in which the renal pedicles were ligated at the times shown. In every case there is a decrease in fractional activity following ligation, and a new, lower, steady state value is finally attained. Kidney ligation, in a control experiment without Prostigmine resulted in no change in the cholinesterase activity.

These curves establish the fact that at least a portion of the normal elimination is via the kidneys. They also prove that mechanisms *other* than renal excretion and destruction by plasma enzyme are operative. The *lowest* infusion rate in these experiments is greater than the maximal destructive capacity of the plasma. If the kidneys were the *sole* disposal mechanism supplementing the enzyme, their exclusion, under these conditions, would result in a continuous accumulation of Prostigmine whose rapidity would depend upon the excess of

<sup>6</sup> We are not unaware of the possible error in equating a rate in moles per liter per minute to one expressed as moles per kilogram body weight per minute. We see no logical alternative to this assumption and have arbitrarily made it throughout this paper.

infusion rate over enzymatic destruction rate. That no such progressive change occurs, that new steady state levels are in fact established, indicates that although the enzyme is incapable of working any faster and the kidneys are excluded, *Prostigmine elimination is proceeding nevertheless by other routes.*

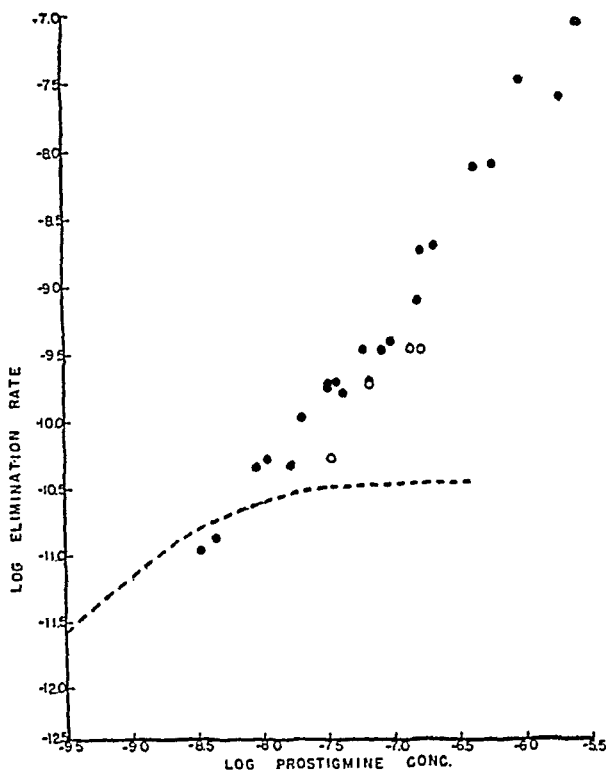


FIG. 7. RELATIONSHIP OF ELIMINATION RATE TO THE STEADY STATE PROSTIGMINE LEVEL IN DOGS

Ordinates: Moles per kilogram per minute. Abscissae: Moles per liter. The broken line represents the contribution of plasma destruction to the total elimination. The four open circles represent kidney ligation experiments.

The contribution of the kidneys to the overall elimination of Prostigmine can be evaluated by two independent means—determination of the renal clearance by usual methods, and calculation of the deficiency in expected overall elimination when the kidneys are ligated.

The Prostigmine clearance was obtained by continuous infusion of the drug in dogs whose ureters had been catheterized. Glucose (6 per cent) in distilled water was infused intravenously at a rate of 200 cc. per hour to promote an adequate urine volume. After establishment of a steady state the total urine excreted in two successive hourly periods was collected and assayed for Pro-

stigmine.<sup>7</sup> Venous blood samples were taken at the beginning and end of each urine collection period, and their Prostigmine concentrations determined. The results of three such experiments are summarized in table 2. The mean plasma clearance was 99 cc. per minute and the creatinine clearance<sup>8</sup> in one dog at the conclusion of the experiment was 99-119 cc. per minute. These data suggest that Prostigmine is excreted by glomerular filtration.

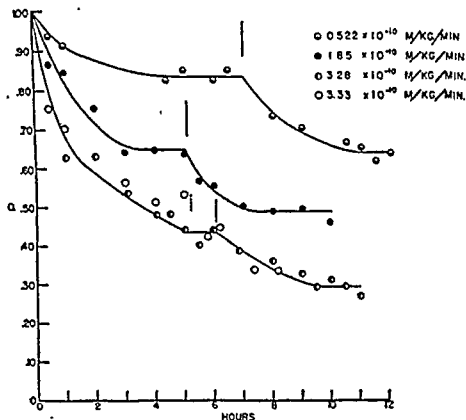


FIG. 8. EFFECT OF KIDNEY LIGATION ON STEADY STATE CHOLINESTERASE ACTIVITIES IN DOGS

The value  
The times at  
The broken

given in the upper right corner.  
vertical lines above each curve.  
which no distinct curve was drawn.

In order to compare these results with those obtained in the kidney ligation experiments, the clearance must first be expressed as a rate constant of excretion,  $k_e$ , in the equation

$$-(dI/dt) = k_e I$$

where  $I$  is the molar inhibitor concentration. The mean value of  $k_e$  from the data of table 2 is  $4.5 \times 10^{-3}$  per minute. The open circles of figure 7 represent the four renal ligation steady states. The difference between the observed and expected elimination rate for each point (i.e. the ordinal distance from the curve of solid circles) should give the contribution of the kidneys at each particular Prostigmine concentration. The calculations, presented in table 3, yield a mean

<sup>7</sup> Appropriate dilutions were added to a standard cholinesterase solution, allowed to stand overnight at 5°, and the resulting activity compared with the normal in the usual way. Normal urine does not inhibit the enzyme.

<sup>8</sup> Kindly determined by Dr. Douglas S. Riggs.

value of  $4.0 \times 10^{-3}$  per minute for  $k_e$ , in satisfactory agreement with that obtained by the clearance method. This agreement is evidence that within the limits of experimental error the sole effect of ligating the renal pedicles is to prevent the excretion of Prostigmine, the kidneys playing no appreciable role in destruction of the drug.

TABLE 2  
Relationship of renal excretion to plasma Prostigmine level in the dog

WT.	IN-FUSION RATE	INFUSION RATE	OBSERVED $q$	MEAN PLASMA PROSTIGMINE CONC.	URINE VOL.	PROSTIGMINE OUTPUT	MEAN PLASMA CLEARANCE	$k_e$	RECOVERY OF PROSTIGMINE
kgm.	mgm./hr.	M/kgm./min.		M/L	cc./hr.	M/hr.	cc./min.	min. <sup>-1</sup>	per cent
25.0	0.488	$9.72 \times 10^{-10}$	0.344 0.322 0.337	$1.26 \times 10^{-7}$	220 353	$6.15 \times 10^{-7}$ $7.83 \times 10^{-7}$	93*	$3.7 \times 10^{-3}$	47.0
18.0	17.6	$4.88 \times 10^{-8}$	0.075 0.061 0.088	$1.00 \times 10^{-6}$	58 48	$5.51 \times 10^{-6}$ $4.38 \times 10^{-6}$	83	$4.6 \times 10^{-3}$	9.5
19.8	17.6	$4.44 \times 10^{-8}$	0.106 0.106 0.106	$2.52 \times 10^{-6} \dagger$	38 198	$1.55 \times 10^{-5}$ $1.55 \times 10^{-5}$	102	$5.2 \times 10^{-3}$	29.4

\* Creatinine clearance in this dog: 99-119 cc./min.

† Determined by diluting serum 1:60 with normal 10% dog serum.

TABLE 3  
Effect of kidney ligation in dogs on the elimination of Prostigmine

$$\frac{\text{Excretion rate}}{\text{Prostigmine conc.}} = k_e$$

(A)	(B)	(C)	(B - C)	$\left(\frac{B - C}{A}\right)$
PROSTIGMINE CONC.	NORMAL ELIMINATION RATE	KIDNEY-LIGATED ELIMINATION RATE	EXCRETION RATE	$k_e$
M/L	M/kgm./min.	M/kgm./min.	M/kgm./min.	min. <sup>-1</sup>
$3.26 \times 10^{-8}$	$1.57 \times 10^{-10}$	$0.5 \times 10^{-10}$	$1.07 \times 10^{-10}$	$3.29 \times 10^{-3}$
$6.15 \times 10^{-8}$	$3.15 \times 10^{-10}$	$2.0 \times 10^{-10}$	$1.15 \times 10^{-10}$	$1.87 \times 10^{-3}$
$1.23 \times 10^{-7}$	$8.9 \times 10^{-10}$	$3.2 \times 10^{-10}$	$5.7 \times 10^{-10}$	$4.63 \times 10^{-3}$
$1.48 \times 10^{-7}$	$13.0 \times 10^{-10}$	$3.0 \times 10^{-10}$	$10.0 \times 10^{-10}$	$6.75 \times 10^{-3}$

Average  $k_e = 4.0 \times 10^{-3}$ .

Average clearance (20 kgm. dog) = 80 cc./min.

Figure 7 and tables 2 and 3 indicate that at very high Prostigmine levels the sum of destruction by plasma and excretion by the kidneys can not account for more than a portion of the total elimination. But at low and moderate levels (less than about  $10^{-7}$  M/L, or an uncorrected enzyme activity no lower than 0.30) these two mechanisms do account for the bulk of the Prostigmine elimination.

The latter conclusion is confirmed by the following experiments. After a steady state had been established, the continuous infusion was stopped and a large sample of arterial blood withdrawn, centrifuged, and the serum incubated at 37°. Periodic determinations of enzyme activity were then made on serum samples from the animal and from the incubated serum. In this way the elimination of Prostigmine in the intact dog could be compared with its simultaneous destruction in serum of the same animal. Figure 9 shows the striking difference between *in vivo* and *in vitro* Prostigmine elimination. When the ex-

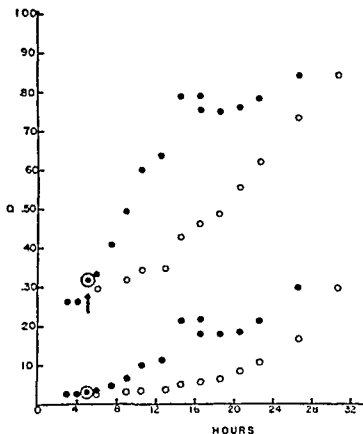


FIG. 9. RECOVERY OF DOG SERUM CHOLINESTERASE ACTIVITY IN VIVO AND IN VITRO AFTER DISCONTINUATION OF PROSTIGMINE INFUSION

Obs . . . . . In vitro . . . . . In vivo . . . . .  
 M/kg. . . . .  
 blood . . . . .

periment was repeated in a renal-ligated animal the curves of figure 10 were obtained, in which the *in vivo-in vitro* difference in elimination rate is practically abolished. At these moderate Prostigmine concentrations therefore, the overall elimination is essentially the sum of renal excretion and plasma destruction.

c. *Hepatic excretion or destruction.* Infusions were made into a small vein of the portal system and steady states established in four dogs. The infusions were then continued at the same rate but shifted to the femoral vein (three experiments). The uncorrected results are shown in figure 11. The *initial* steady states are in all cases at a higher activity than usual. A given portal infusion rate produces the same effect as a slower femoral infusion, as though the liver were steadily intercepting a fraction of the Prostigmine.<sup>9</sup> Moreover, when each

<sup>9</sup> Compare the infusion at  $4.77 \times 10^{-10}$  M/kgm./min. (a pure portal infusion, not shifted to the femoral vein) with the femoral infusion at  $3.78 \times 10^{-10}$  M/kgm./min. in figure 5.



infusion is transferred to the femoral vein, there is a consistent decrease of  $a$  to the usual value.

Obviously, the liver takes part in the elimination of Prostigmine. We have been able to detect appreciable Prostigmine activity in the bile, which normally does not inhibit cholinesterase, but how much of the hepatic contribution is excretory and how much metabolic is not evident. Furthermore, the total contribution of the liver is far too small to explain the rapid elimination of Prostigmine at very high plasma concentrations. One must assume from figure 7 that steady states would result from continuous infusion even if liver and kidneys were excluded and the maximal destruction rate by plasma exceeded. The

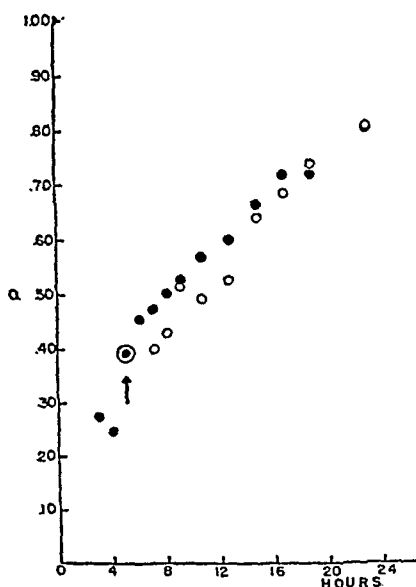


FIG. 10. RECOVERY OF SERUM CHOLINESTERASE ACTIVITY OF RENAL-LIGATED DOGS IN VIVO AND IN VITRO AFTER DISCONTINUATION OF PROSTIGMINE INFUSION

○ In vitro      ● In vivo

The values of  $a$  are uncorrected. The infusion ( $2.12 \times 10^{-6}$  M/kgm./min.) was stopped at the time indicated by the arrow. A large sample of arterial blood withdrawn at this time was centrifuged and the serum promptly incubated.

nature of the unidentified disposal mechanisms remains unknown but one might surmise that esterases of various fixed tissues play an important role.

3. *Prostigmine elimination during the recovery phase.* In six experiments blood samples were withdrawn periodically after stopping a continuous infusion, the steady state having first been established. The course of recovery of the plasma cholinesterase activity is plotted in figure 12. The initial points correspond to the steady state values previously established at the infusion rates indicated for each curve. The data are uncorrected. The observed course of recovery in every case is fully predictable from the known rate of Prostigmine elimination

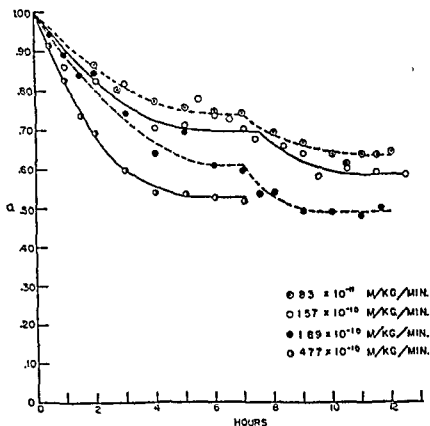


FIG. 11. ESTABLISHMENT OF STEADY STATE CHOLINESTERASE ACTIVITIES BY CONTINUOUS INFUSION OF PROSTIGMINE INTO THE PORTAL SYSTEM OF DOGS

The values of  $a$  are uncorrected. Infusion rates are given in the lower right corner. After seven hours the infusions, in three of the experiments, were transferred to the femoral vein and continued at the same rate.

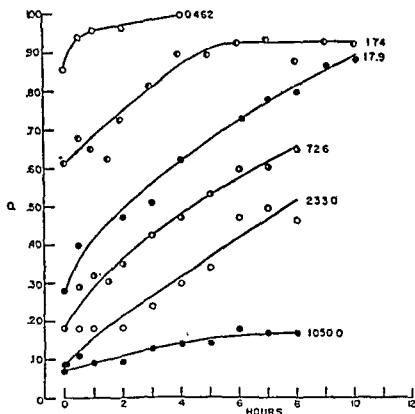


FIG. 12. RECOVERY OF SERUM CHOLINESTERASE ACTIVITY IN DOGS AFTER DISCONTINUATION OF PROSTIGMINE INFUSIONS

The values of  $a$  are uncorrected. Figures at the right of each curve refer to the rates ( $M \times 10^{-10}/\text{kgm./min.}$ ) of the infusions which were discontinued at zero time.

at each level of enzyme activity (figure 6) and the known increase in activity with a given change in Prostigmine concentration (figure 1).

*Intravenous administration of single doses.* In the well controlled steady states resulting from continuous infusion all the tissues are presumably at equilibrium with respect to Prostigmine concentration. What is the course of the plasma Prostigmine level after single intravenous doses, and how quickly is tissue equilibrium established? In the following experiments the animals were not anesthetized.

The single injection of 25 microgm.<sup>10</sup> per kgm. into a 20 kgm. dog (0.5 mgm. actual dose) resulted in an immediate reduction of activity to less than 0.0%. The time course of recovery is given in table 4. This single dose depresses enzyme activity below 25 per cent of normal for six hours and some Prostigmine would obviously persist for more than twenty-four hours. Comparison of the

TABLE 4

*Recovery of plasma cholinesterase activity in the dog after intravenous injection of 0.5 mgm. Prostigmine methylsulfate*

TIME	OBSERVED $a$	CORRECTED $a$
<i>hours</i>		
0	0.23	0.015
1	0.49	0.065
2	0.64	0.115
3	0.65	0.120
4	0.73	0.165
5	0.80	0.225
6	0.82	0.250

corrected and uncorrected figures in the table shows how failure to correct the data would have distorted both the intensity and duration of the drug action.

In figure 13 are shown the uncorrected results of three experiments in which a single intravenous dose was followed by repeated determinations of serum activity. These curves show an immediate attainment of maximal Prostigmine concentration, an initial phase of very rapid recovery during the first twenty minutes, and a subsequent slower recovery rate. If a dose of 0.5 mgm. in a 20 kgm. dog remained entirely within the plasma water (assumed to be 5 per cent of body weight), the Prostigmine concentration would be  $1.5 \times 10^{-6}$  M/L and the immediate uncorrected activity would be 0.10. If the same dose were then distributed uniformly throughout *all* body water (65 per cent of body weight), the concentration would be  $1.15 \times 10^{-7}$  M/L and the uncorrected activity would be 0.35. The two lowest curves of figure 13 are therefore reasonably consistent with the hypothesis that following intravenous injection Prostigmine is first contained within the plasma water and then distributed during the subsequent twenty minutes throughout a considerable volume of extravascular water. The

<sup>10</sup> Unless otherwise stated, all doses are in terms of Prostigmine methylsulfate.

recovery rate after the first twenty minutes is comparable in all respects to that seen after discontinuing an infusion (cf. figure 12) and the same modes of elimination are presumed to be operative.

*Oral administration of single doses.* Doses of 15 and 50 mgm. Prostigmine methylsulfate were given to two dogs, either in solution by stomach tube or in Konseal capsules by mouth. The dogs had previously been fasted for at least twelve hours and were not fed during the experimental period. No anesthesia was employed but atropine sulfate was included with the higher dose.

The uncorrected results of determinations of serum cholinesterase activity in seven such experiments are seen in figure 14. The peak effect occurred be-

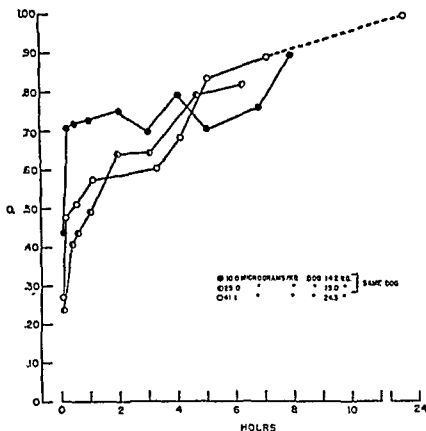


FIG. 13. EFFECTS OF PROSTIGMINE BY SINGLE INTRAVENOUS DOSE UPON THE SERUM CHOLINESTERASE ACTIVITY OF DOGS

The values of *a* are uncorrected

tween 30 minutes and 4 hours depending upon the dose and the vehicle. It was reached sooner with the large than with the small dose. In one of the two dogs a given dose always produced a lower activity than in the other, presumably reflecting an individual difference in absorption.

Doses of this order produced a profound inhibition of the serum cholinesterase whose activity was in all cases depressed below 0.09. Nevertheless, except for salivation and hyperperistalsis, which were readily controlled by atropine, the animals seemed quite normal throughout the experiments. Fasciculation<sup>11</sup> only occurred in the single experiment where 50 mgm. were given in solution.

<sup>11</sup> The phenomenon referred to here is a generalized fasciculation of muscle groups at a distance from the site of injection.

From an activity of about 0.03 (0.33 uncorrected) the recovery period was at least 24 hours. The time course of recovery was entirely comparable with that observed after continuous infusions and single intravenous injections.

An oral dose of 15 mgm. in solution produced a peak effect of about the same magnitude as 0.5 mgm. intravenously. This is in accord with clinical observation of the unusual discrepancy between oral and parenteral dosage. Consider the 15 mgm. dose which produced a minimal activity of 0.288 in two hours (figure 14, upper graph). To *establish* this response by single intravenous injection would require about 0.5 mgm. To *establish and maintain* the same level

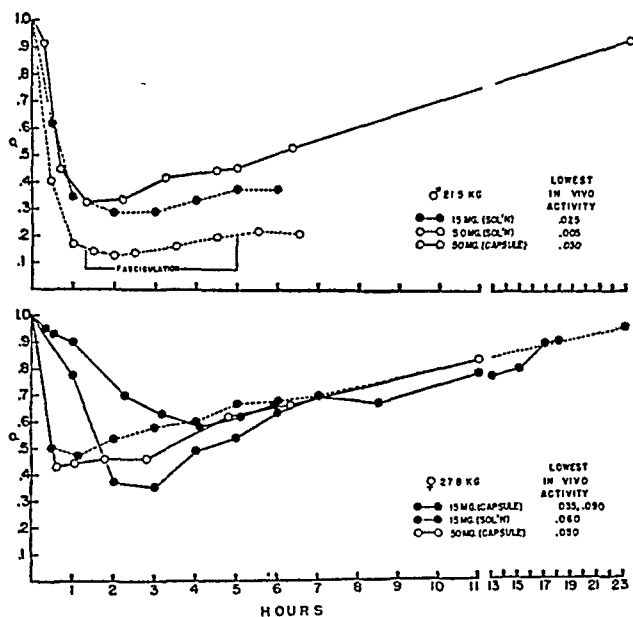


FIG. 14. EFFECT OF SINGLE ORAL DOSES OF PROSTIGMINE UPON THE SERUM CHOLINESTERASE ACTIVITY OF DOGS

The values of *a* are uncorrected. Lowest corrected *in vivo* activities attained are indicated on the right. In lower part there are two experiments with 15 mgm. (capsule).

by continuous infusion would require a rate of  $10^{-9}$  moles per kgm. per min., or 0.43 mgm. per hour. (The difference between portal and femoral infusion is not great enough to affect this argument.) It is obvious that were the drug being absorbed from the gastrointestinal tract at this rate, the activity would remain at 0.288. Were an amount greater than 0.5 mgm. absorbed initially or in any hour, the activity would be reduced *below* this level. Finally, were absorption of the 15 mgm. dose extended over many hours, in hourly amounts even approaching this, the recovery period would be significantly prolonged. That none of these hypothetical events occurred forces one to the conclusion that the bulk of an oral dose is not absorbed at all.

*Pharmacological actions of Prostigmine.* Many investigators have attempted to correlate changes in physiological function seen in Prostigmine administration

with the degree of inhibition of plasma cholinesterase. These attempts have been uniformly misleading because of neglect of the dilution effect. Thus salivation, miosis, intestinal hyperactivity and even fasciculation of the skeletal musculature have been reported to occur with only moderate depression of the plasma cholinesterase activity. When such findings are properly corrected they prove to be consistent with our own observations, namely, that *no physiological responses appear until the plasma cholinesterase is at least 90 per cent inhibited*. Kraye, Goldstein and Plachte (6) showed that with physostigmine the various physiological effects, beginning with intestinal hyperactivity, are manifested only at corrected plasma cholinesterase activities below 50 per cent of normal. Because of the different potencies of Prostigmine and physostigmine, however, molar concentrations are approximately the same when the plasma enzyme is inhibited 90 per cent by the former or 50 per cent by the latter.

In our experiments atropine was routinely included to control parasympathetic actions whenever high Prostigmine levels were expected. In certain cases, however, it was omitted. In six continuous infusions no atropine was given. The highest infusion rate was  $7.56 \times 10^{-10}$  moles per kgm. per min. producing a steady state activity of 0.028. In two single intravenous experiments in which no atropine was used the lowest activity attained was 0.023. Despite inhibition of 97 per cent of the plasma cholinesterase activity in these eight experiments there was no excessive salivation, hyperperistalsis, urination or miosis. When greater inhibition was produced atropine was always present, so it is impossible to conclude at what precise points various parasympathetic effects might have appeared.

When a 15 mgm. dose was given orally and atropine omitted, the results were rather variable. In two dogs, salivation, gastrointestinal hypermotility and miosis were observed at activities of 0.09 and 0.025. In a third animal at an activity of 0.035 defecation alone was observed. A fourth dog displayed no obvious parasympathetic effects although plasma cholinesterase activity was as low as 0.06.

The continuous infusion of Prostigmine produced no significant change in the heart rate regardless of the infusion rate or level attained. Here too, however, atropine at the higher infusion rates may have prevented the action. In the range  $1.34 \times 10^{-11}$  to  $7.56 \times 10^{-10}$  M/kgm./min., where parallel experiments were performed with and without atropine, no significant differences in heart rate could be detected.

In the continuous infusions, at rates just sufficient to produce a steady state activity of 0.025, fasciculation appeared at 0.03 and persisted all the while the activity remained below this point. If a faster infusion was employed the activity could quickly reach a point as low as 0.005 before fasciculation appeared; then it persisted and upon stopping the infusion did not disappear until the activity had increased to 0.03 again. In no experiment resulting in a steady state activity higher than 0.03 was any fasciculation observed.

A curious effect was noted at the two highest infusion rates. Fasciculation began at once (the activity had already been reduced to 0.004) but shortly

diminished in intensity and disappeared despite the low steady state activity of 0.003. After the infusion had been stopped severe fasciculation *reappeared* at a level of 0.004 and persisted during the recovery period. These experiments were discontinued after six hours of recovery, plasma enzyme activity having risen to 0.01 and fasciculation continuing. The finding that Prostigmine *produces* fasciculation at one concentration and *inhibits* fasciculation at a higher concentration would seem consistent with the known effects of optimal and excessive acetylcholine concentration at the muscle end-plate.

Fasciculation was not observed in any of the single intravenous experiments although the activity after distribution of Prostigmine to the extravascular fluid reached as low as 0.04. When the largest dose (50 mgm.) was given by mouth, fasciculation appeared when the activity fell to 0.01, persisted while it fell still

TABLE 5

*Fasciculation in the dog after 50 mgm. Prostigmine methylsulfate by mouth*

TIME	OBSERVED $\sigma$	CORRECTED $\sigma$	FASCICULATION
<i>min.</i>			
0	1.00	1.00	0
41	0.41	0.045	0
57	0.17	0.010	0
75			+
87	0.14	0.006	+
117	0.13	0.005	+
147	0.14	0.006	+
207	0.16	0.009	+
267	0.20	0.013	+
327	0.22	0.015	0
387	0.22	0.015	0

further to 0.005, and disappeared during recovery at 0.014. These results are shown in table 5 and in the upper section of figure 14.

Thus fasciculation of the skeletal musculature never occurred when the plasma cholinesterase activity was higher than 0.03. To produce this degree of enzyme inhibition in the plasma requires  $1.2 \times 10^{-7}$  M/L Prostigmine, which may therefore be considered the minimal plasma Prostigmine level for the occurrence of fasciculation.

It should be evident from the above that inhibition of the plasma cholinesterase *per se* is not the cause of any observed physiological effects since the constant absence of such effects has been demonstrated in spite of nearly complete inhibition of this enzyme. This is in agreement with the observations of Hawkins and Gunter (12) and of Mazur and Bodansky (13) who found that the plasma enzyme could be almost fully inhibited without apparent consequence. The latter authors showed further that in di-isopropylfluorophosphate inhibition, toxic manifestations could be related to inhibition of tissue but not plasma cholinesterase.

These findings become intelligible in the light of the fact that tissue cholin-

esterases are less sensitive than the plasma enzyme to inhibition by Prostigmine. The consequence of this sensitivity difference can be illustrated by the behavior of cholinesterase of intact dog erythrocytes in the presence of Prostigmine.

Fresh blood was oxalated, centrifuged and the plasma discarded. The cells were washed three times in ten volumes of saline and finally suspended in ten volumes of the usual bicarbonate-Ringer solution, containing varying amounts of Prostigmine. After standing overnight at 5°, the determinations were performed as usual except that the acetylcholine concentration was one-tenth that previously employed. At this substrate concentration uninhibited cell suspensions gave a maximal hydrolysis rate, which was 1.0 mM acetylcholine per liter packed cells per minute.

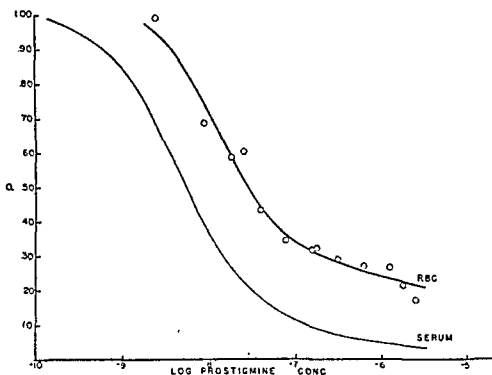


FIG. 15. INHIBITION OF INTACT ERYTHROCYTE CHOLINESTERASE OF THE DOG BY PROSTIGMINE

RBC: Erythrocyte suspension, 10%. The curve of best fit to the observed dog serum activities of figure 1 is shown for comparison.

Figure 15 shows the inhibition curve thus obtained and, for comparison, the best curve through the observed points of figure 1 (serum inhibition). Neglecting competition, apparent dissociation constants are  $5.4 \times 10^{-9}$  for serum,  $2.8 \times 10^{-8}$  for erythrocytes. The two curves, confirming the results of previous investigators, show that for a given degree of inhibition the erythrocyte enzyme requires considerably more Prostigmine than the plasma enzyme. If, for example, plasma and erythrocyte cholinesterases were to be separated by a semi-permeable membrane, in the presence of  $10^{-7}$  M/L Prostigmine, the activity of the former would be 0.10, that of the latter 0.35. If the tissue cholinesterases whose inhibition must be responsible for observed physiological responses to Prostigmine are even less sensitive to the drug than the erythrocyte enzyme, this differential effect of a given inhibitor concentration would be even more pronounced. Thus a rise in Prostigmine level sufficient to cause a small reduction in plasma activity (from 0.05 to 0.01) would induce a disproportionate



change (from 0.72 to 0.28) in the activity of a tissue enzyme one-fiftieth as sensitive to the drug.

### III. Prostigmine levels and cholinesterase inhibition in patients with myasthenia gravis.

It was the purpose of the following experiments to determine whether the principles established in dogs could be applied in the therapeutic use of Prostigmine in patients with myasthenia gravis. In particular we wished to discover whether plasma Prostigmine levels could be reliably established, and if so, what relation these levels bore to therapeutic responses; whether the mechanisms for elimination of Prostigmine were similar to those of the dog; and whether the

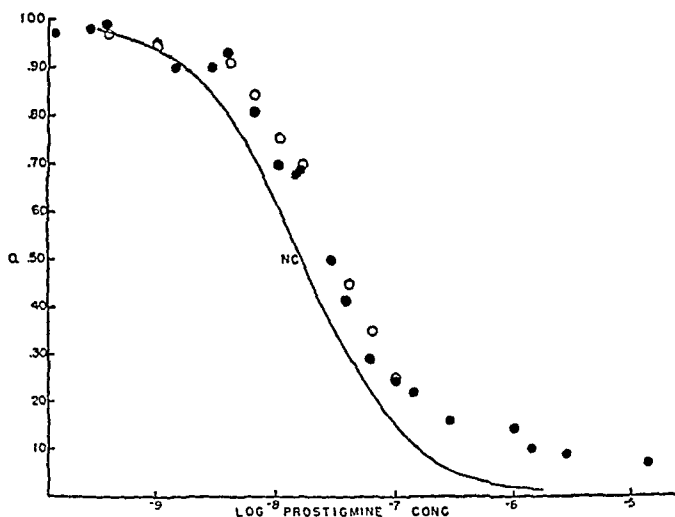


FIG. 16. INHIBITION OF HUMAN SERUM CHOLINESTERASE BY PROSTIGMINE

● Normal subjects                      ○ Myasthenic patient

Serum concentration 10%. NC is the calculated non-competitive curve in the absence of acetylcholine.

large oral doses required led to the same plasma levels as smaller parenteral doses.

The methods employed were those described in the previous sections. Prostigmine methylsulfate was infused at 27 different rates in a total of twelve patients on whom the diagnosis of myasthenia gravis had been made beyond reasonable doubt. All required Prostigmine regularly in oral doses up to 450 mgm. (as the bromide) daily. Prior to each experiment the drug was withheld for as long a period as possible (2 to 40 hours) and the plasma cholinesterase activity then determined just before each infusion was started. The uninhibited activity, taken as the normal for calculating degree of inhibition, was obtained by incubating serum at 38° for 24 to 48 hours. This suffices to restore full enzyme activity by destroying the Prostigmine; yet the same period of incubation does not significantly reduce the normal cholinesterase activity in the absence of Prostigmine. Infusions were made into a forearm vein, the drug being contained in a saline solution with 5 per cent glucose in a standard hospital intravenous drip apparatus calibrated in drops per minute.

Except in three experiments at low infusion rates, atropine sulfate was included in the amount of 0.1 mgm per mgm Prostigmine methylsulfate which proved sufficient to prevent toxic parasympathetic effects

In order to translate observed inhibition to plasma Prostigmine concentration it was necessary to establish an *in vitro* inhibition curve corresponding to the dog serum curve of figure 1. This is seen in figure 16, where the solid circles represent serum from three normal subjects (two male, one female) and the open circles serum of a myasthenic patient (A. J. B., female). The curves obtained are identical, within experimental error, for male and female, and for myasthenic and normal serum. This is in accord with the findings of other investigators.

TABLE 6

*Continuous infusion of Prostigmine methylsulfate in a myasthenic patient*

J. V. Male 62 kgm

Regular dose 450 mgm Prostigmine bromide daily

Last dose 30 mgm at -7 hours

Normal serum cholinesterase activity 240 mM per liter per hour

11 27-45 INFUSION RATE 29 $\gamma$ /KGM /HR WITH ATROPINE SULFATE 2.9 $\gamma$ /KGM /HR			11 29-45 INFUSION RATE 100 $\gamma$ /KGM /HR WITH ATROPINE SULFATE 10 $\gamma$ /KGM /HR		
Time	Observed a	Remarks	Time	Observed a	Remarks
<i>min</i>			<i>min</i>		
0	0.834	Start infusion	-9	0.629	
60	0.585		0		Start infusion
107		Fasciculation	40		Fasciculation
117	0.526	Fasciculation	66	0.444	Fasciculation
182	0.562	Fasciculation	120	0.410	Fasciculation
241	0.485	Fasciculation	180	0.390	Fasciculation
344 } 0 }		Stop infusion	241	0.419	Fasciculation
60	0.741	No fasciculation	309	0.389	Fasciculation
113	0.976		312 } 0 }		Stop infusion
			61	0.534	Fasciculation continues
			117	0.581	No fasciculation

The competition and dilution effects are seen with human as with dog serum, the non competitive curve is shown as the solid line of figure 16. It will be observed that human plasma cholinesterase is about four times less sensitive than the dog enzyme to inhibition by Prostigmine. In dog serum the dissociation constant of the enzyme-Prostigmine complex is  $3.89 \times 10^{-3}$ , the constant here is  $1.6 \times 10^{-3}$ . Figure 16 is used to correct the experimental data to *in vivo* conditions and to convert fractional activity to Prostigmine concentration.

In myasthenic patients, as in the dog, steady state levels could be established by infusion of the drug at a constant rate. Data from two representative experiments are summarized in table 6 and a typical protocol is reproduced in table 7. If the data of these tables be plotted graphically it will be apparent

that the time course of the establishment of levels is entirely comparable to that seen in the dog experiments (cf. figure 5).

At the start of an infusion the cholinesterase activity was, as a rule, below normal; some of the Prostigmine last ingested had not been completely elimi-

TABLE 7

*Effects of Prostigmine infusion upon manifestations of myasthenia gravis*

A. J. B. Female. 50 kgm. 1-28-44.

Regular dose: 360 mgm. Prostigmine bromide daily.

Last dose: 15 mgm. at 12 midnight.

Normal serum cholinesterase activity: 25S mM per liter per hour.

Calibration: 44 drops per min. = 3 cc. per min.

Infusion solution: Prostigmine methylsulfate 10 mgm., atropine sulfate 1 mgm. in 1500 cc. 5 per cent glucose in saline.

TIME	OBSERVED $\alpha$	DROPS PER MINUTE	REMARKS
9:30 A.M.	0.80		Feels very weak. Cannot wrinkle brow or purse lips. Bares teeth poorly. Eye-closing weak. Grip poor.
9:55			Infusion started. 3 cc./min. = 24 $\gamma$ /kgm./hr. or $1.20 \times 10^{-9}$ M/kgm./min.
10:00		40	
10:11		41	Considerable improvement in eye-closing, tongue-protrusion. Grip slightly stronger.
10:13		44	
10:55	0.565	43	1 hour. All tests much improved. Can whistle.
11:55	0.500	44	2 hours. Eating lunch. Feels strong. Mild griping.
1:05 P.M.	0.510	43	3 hours, 10 min. Griping passed off. Ate without difficulty, then talked a great deal. Now her tongue feels tired and speech is a little thick. Eye-closing and masseter strength remain good. No fasciculation.
1:55	0.494	44	4 hours. Speech cleared up on resting.
2:55	0.508	43	5 hours. Strength tests and subjective state remain good. Infusion stopped.
3:25	0.587		30 mins. Can no longer purse lips. Eye-closing may be a little weaker.
3:55	0.705		1 hour. All tests weaker now. Eye-closing much weaker.
4:25	0.855		1 hour, 30 mins. Feels very weak but not as weak as at 9 A.M. All tests about the same as before infusion.
4:55	0.850		2 hours.

nated. In the patient with the clinically mildest disease, who was on the smallest regular dosage, the drug had been withheld for 40 hours and the cholinesterase activity had returned to normal by the start of the experiment. Since most patients could not forego Prostigmine for many hours, the initial uncorrected enzyme activities varied between 1.0 and 0.60.

Despite the residual Prostigmine level, the steady state activities reached with a given infusion rate were always higher (less inhibition) than in the dog, as illustrated by the data of table 8. These differences can be explained fully by the difference in sensitivity of the two plasma enzymes.

In figure 17 steady state activities (uncorrected) have been plotted against infusion rates, and the best line through the points is shown corrected to *in vivo*

TABLE 8

*The effect of Prostigmine infusion rate upon serum cholinesterase activity in dogs and myasthenic patients*

RATE	DOGS		PATIENTS	
	Observed <i>a</i>	Corrected <i>a</i>	Observed <i>a</i>	Corrected <i>a</i>
<i>M/kgm./min.</i>				
$1 \times 10^{-10}$	0.76	0.175	0.90	0.44
$1 \times 10^{-9}$	0.31	0.025	0.55	0.165

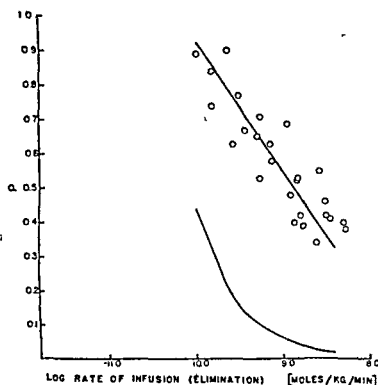


FIG. 17. RELATIONSHIP OF STEADY STATE CHOLINESTERASE ACTIVITY TO RATE OF PROSTIGMINE INFUSION IN MYASTHENIC PATIENTS

The points represent uncorrected values of *a*. There are two identical points at  $(-8.80, 0.42)$ . The curve of best fit is shown corrected to *in vivo* conditions in the lower curve.

conditions. Thus actual cholinesterase activities were reduced to about 0.45 at the lowest and 0.02 at the highest infusion rate. The rates employed were in the range  $1.0-52.0 \times 10^{-10}$  M/kgm./min. or 0.1-6.5 mgm. per hour. Comparison with the analogous figure 6 reveals that any infusion rate produces a greater inhibition of the plasma cholinesterase in the dog than in the patient. Here a steady state activity is predictable from the infusion rate with an approximate standard error of  $\pm 0.087$  (cf. footnote 2). The unusual scatter of

the points in figure 17 can not be attributed with certainty to a patient-to-patient variation. Possible sources of error are the presence of differing concentrations of previously absorbed Prostigmine, and the practical impossibility of maintaining every infusion long enough to be certain a true steady state equilibrium is reached.

While there is a striking difference in the enzyme activities established in dogs and in patients, the Prostigmine concentrations are actually the same. Figure 18 shows (solid circles) the rates of infusion (or elimination) plotted against Prostigmine concentration in patients, and on the same figure (open circles) a portion of the analogous figure 7 (dogs). For all practical purposes the two sets of data are identical.

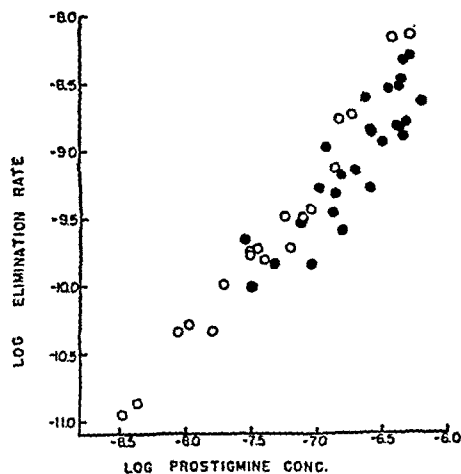


FIG. 18. RELATIONSHIP OF ELIMINATION RATE TO THE STEADY STATE PROSTIGMINE LEVEL IN MYASTHENIC PATIENTS

Ordinates: Moles per kilogram per minute. Abscissae: Moles per liter.  
 ● Myasthenic patients. ○ Dogs (from figure 7)

The curves presented here enable one to understand the wide range of doses required in clinical practice. If the dose of Prostigmine is doubled successively, for example, the plasma Prostigmine level also increases geometrically (figure 18), but per cent inhibition of the plasma enzyme only increases by arithmetic increments (figures 16, 17). This phenomenon is not peculiar to the plasma cholinesterase. It is implicit in any curve of enzyme inhibition which assumes a sigmoid shape on a semi-logarithmic plot.

We have shown that a given infusion rate establishes a predictable steady state drug level which is the same in the myasthenic patient as in the dog. Conversely, at a given plasma Prostigmine concentration the overall elimination of the drug proceeds at the same rate in patient and dog. This suggests that the various disposal mechanisms may be the same in both cases, but direct proof would be required. Furthermore, it is not known whether *normal* human subjects would yield similar curves.

During the course of the infusions all the patients showed improvement in their status with respect to facial expression, ability to swallow, voluntary muscle strength, and sense of well-being (see table 7). But the level at which improvement occurred varied from patient to patient. Those who had required the largest daily doses for control of their symptoms and whose weakness was the most severe, also needed a higher plasma Prostigmine level (lower  $a$ ) for the relief of symptoms during infusions. For example, the patient (J. V.) who had taken 450 mgm. daily was unable to speak without slurring at the start of the experiment, although his plasma cholinesterase activity was only 0.10. When the steady state level of 0.025 was reached his symptoms were much improved.

In three infusions without atropine, levels of 0.07, 0.065, and 0.035 were reached without undesirable parasympathetic actions or discomfort of any kind. In a fourth infusion, atropine had to be given to relieve severe griping abdominal cramps at a level of 0.04. In the remaining experiments atropine was included routinely so it is impossible to fix definite levels at which the various undesirable effects of Prostigmine might have occurred. It was our impression that intestinal hyperactivity is first to occur, in the absence of atropine, but only when the Prostigmine level is high enough to reduce the plasma activity below 0.10.

The relation of fasciculation to plasma Prostigmine concentration was consistent in each patient but variable from individual to individual. Fasciculation occurred in six different experiments. The highest activity at which it was observed was 0.065, corresponding to a Prostigmine concentration of  $2.7 \times 10^{-7}$  M/L. (cf.  $1.2 \times 10^{-7}$  M/L in the dog.)

We were interested in the clinical implications of the finding that Prostigmine administered orally to the dog is largely unabsorbed. It was possible to show that this was also true in a patient who was taking 330 mgm. Prostigmine bromide daily by mouth prior to thymectomy and was then maintained post-operatively by intravenous infusion of 12 mgm. Prostigmine methylsulfate daily. In the preoperative period the plasma Prostigmine levels were just detectable by our assay method. The lowest activity recorded was 0.18 (0.806 uncorrected), corresponding to  $8.15 \times 10^{-8}$  moles per liter serum. This is the level to be expected from the continuous infusion of 6.5 microgm. per kgm. per hr., or 7.2 mgm. per day in this particular patient. Evidently no more than one-thirtieth of the dose entered the blood stream. Of the 330 mgm. actually ingested, only 1.1 mgm. could be found in the 24-hour urine, and 2.5 mgm. in the stool. Further, since fecal Prostigmine content was negligible, the material must have been largely inactivated in the gut. The sole alternative possibility, that this patient might somehow have required an extraordinarily high absorption of the drug to produce the observed degree of inhibition, was ruled out on the first post-operative day when the predicted plasma Prostigmine level ( $1.1 \times 10^{-7}$  M/L) and activity 0.15 (0.753 uncorrected) resulted from an infusion rate of 0.5 mgm. per hour. On this day 3.54 mgm. were detected in the urine. The renal excretion rate proved closely comparable to that found in the dog, the clearance being approximately 100 cc. per minute.

## SUMMARY AND CONCLUSIONS

I. The behavior of the cholinesterase-Prostigmine-acetylcholine system was investigated in the serum of dogs, normal humans and myasthenic patients, and in dog erythrocytes.

1. Prostigmine inhibits both human and dog serum enzymes and the inhibition is competitive with respect to acetylcholine.
2. Dog serum is more sensitive to the drug than is human serum; the respective dissociation constants are  $3.89 \times 10^{-9}$  and  $1.6 \times 10^{-8}$ .
3. The cholinesterase of intact dog erythrocytes is about seven times less sensitive to Prostigmine than is the dog serum enzyme. The dissociation constant is somewhat smaller than  $2.8 \times 10^{-8}$ .
4. Serum cholinesterase from the myasthenic patient behaves like that of normal subjects with respect to hydrolysis of acetylcholine and inhibition by Prostigmine.
5. Prostigmine is destroyed enzymatically. Its turn-over number is  $>0.0018$  per minute, that of acetylcholine  $> 100000$  per minute.
6. By measuring serum cholinesterase inhibition one can assay Prostigmine at a minimal concentration of 0.006 micrograms per cc. in dog serum and 0.024 microgm. per cc. in human serum.

II. Prostigmine was administered to dogs by continuous intravenous infusion, and in single doses intravenously and by mouth.

1. Constant, predictable inhibition of serum cholinesterase, representing definite Prostigmine levels, can be produced by infusing the drug at a fixed rate.
2. Disposal of the drug depends upon its concentration in the body fluids. At low levels it is largely destroyed by the plasma and partially excreted by the kidneys. At intermediate levels renal excretion plays a predominant role. At high levels other elimination factors come into play; these include excretion by the liver, and probably destruction by fixed tissue cholinesterases.
3. Renal excretion of Prostigmine is linearly dependent upon the plasma level, and the clearance rate is about 100 cc. per minute. Prostigmine is therefore probably excreted passively by glomerular filtration.
4. Administered orally, only a fraction of the total Prostigmine is absorbed. The remainder may be inactivated in the gastrointestinal tract.
5. Physiological changes do not appear until the Prostigmine level is high enough to produce almost complete inhibition of the plasma enzyme. At these levels, however, tissue cholinesterases are not inhibited to a comparable degree.

III. Prostigmine was administered by continuous intravenous infusion to patients with myasthenia gravis.

1. As in the dog, predictable Prostigmine levels can be achieved by infusion at a constant rate. The relation of drug level to infusion rate, the overall elimination rate, and the renal clearance are approximately the same as in the dog.

2. To depress the plasma cholinesterase activity in equal small steps requires dose increments whose magnitude increases exponentially. This should also be true of the inhibition of tissue cholinesterases. The very wide range of doses required clinically can thus be explained.
3. Parasympathetic side-effects (in the absence of atropine) and fasciculation of skeletal muscle do not appear until the plasma cholinesterase is almost completely inhibited.
4. Objective and subjective improvement in each myasthenic patient appears to require a definite plasma Prostigmine level, which is determined by the severity of his disease.
5. As in the dog, Prostigmine is only partially absorbed when administered orally. Plasma Prostigmine levels explain the clinical finding that an oral dose has the effect of about one-thirtieth its weight given parenterally. Limited data show that recovery from the stool is negligible. Consequently the drug is largely inactivated in or by the gastrointestinal tract.
6. The inhibition of plasma cholinesterase is a convenient indicator of the Prostigmine concentration. It is quite clear that inhibition of this plasma enzyme bears no causal relationship to the pharmacological actions of Prostigmine. We suggest, however, on the basis of the data presented here, that an effective Prostigmine concentration at the neuro-muscular junction demands a definite corresponding drug level in the circulating plasma, and that this level may be measurable by its effect upon the plasma cholinesterase.

We are indebted to Dr. Oliver Cope and Dr. Henry R. Viets for their cooperation in making patients available for study. It is a pleasure to acknowledge the skilled technical assistance of Mr. Henry W. George and Mr. Louis Harris.

#### REFERENCES

1. JUCKER, P.: Schweiz. Med. Wchnschr., **73**, 896, 1943.
2. ODOM, G., RUSSEL, C. K., AND McEACHERN, D.: Brain, **66**, 1, 1943.
3. WILSON, A., AND STOVER, H. B.: Lancet, **1**, 429, 1944.
4. STRAUS, O. H., AND GOLDSTEIN, A.: J. Gen. Physiol., **26**, 559, 1943.
5. GOLDSTEIN, A.: J. Gen. Physiol., **27**, 529, 1944.
6. KRAYER, O., GOLDSTEIN, A., AND PLACHTE, F. L.: THIS JOURNAL, **80**, 8, 1944.
7. ROOT, M. A.: Fed. Proc., **5**, 199, 1946.
8. AMMON, R.: Arch. f. d. ges. Physiol., **233**, 486, 1934.
9. EASSON, L. H., AND STEDMAN, E.: Proc. Roy. Soc. Lond., B, **121**, 142, 1936.
10. ELLIS, S., PLACHTE, F. L., AND STRAUS, O. H.: THIS JOURNAL, **79**, 295, 1943.
11. GOLDSTEIN, A.: Unpublished data.
12. HAWKINS, R. D., AND GUNTER, J. M.: Biochem. J., **40**, 192, 1946.
13. MAZUR, A., AND BODANSKY, O.: J. Biol. Chem., **163**, 261, 1946.



# INFLUENCE OF STRIATED MUSCLE ACTIVITY ON THE LETHAL DOSE OF K-STROPHANTHOSIDE<sup>1</sup>

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The preeminence of the cardiac actions of digitalis bodies is to be attributed to phenomena of two different kinds: to a specific sensitivity of cardiac muscle to the pharmacological actions of the glycosides, and to a selective storage by the heart. The intervention of the latter factor, denied by Hatcher and Eggleston (1), has received experimental support. The heart, which represents less than one per cent of body weight, takes up approximately ten per cent of administered digitalis bodies (2). Moreover, the dose required to produce effects on the isolated heart is much greater than the amount, assuming a uniform distribution of the drug, to which the myocardium would be exposed after an effective dose in the intact animal (3).

Despite many theories, the cause of the selective storage of digitalis bodies by the heart has not been settled. Without excluding the existence of chemical (4) or physical (5) affinities intrinsic to the myocardium, it is necessary to analyze other conditions peculiar to the heart which might contribute to the explanation of its selectivity. For example, the rich blood supply to the heart might result in its receiving a greater amount of drug. This is corroborated by Weese's evidence (6) that the kidney, whose abundant blood supply is comparable to that of the heart, takes up an amount of digitalis glycoside per gram of tissue equal to that stored by the heart.

In the course of a study of the actions of k-strophanthoside on mammalian striated muscle (7), del Pozo observed that the amounts of drug tolerated by cats on intra-arterial injection were greater than the lethal doses calculated from the results ordinarily obtained with Hatcher and Brody's method (8) of bio-assay. As this method calls for the intravenous injection of the drug, it seemed to us that the fact that in our experiments the drug first passed through the peripheral tissues might determine a greater storage of the glycosides by these tissues, and therefore a greater tolerance of the animal (9). However, the additional fact that in those experiments some of the skeletal muscles were active suggested the possibility that the work of the muscles might increase their capacity for storage. Such an hypothesis would have a bearing on the selective storage of digitalis bodies by the heart, which is in constant activity. This paper describes the results of experiments in which the influence of the route of injection and of muscular work on the lethal dose of k-strophanthoside is analyzed.

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**METHODS.** The lethal dose of k-strophanthoside solution was determined on cats weighing between 2 and 3 kgm. The majority of the animals were males (69 in a series of 80). The few females used were neither pregnant nor in puerperium. The animals selected were all apparently healthy. Those which were too fat or too thin were discarded. The cats, after fasting for approximately twenty-four hours, were anesthetized with 0.7 cc. of Dial (Ciba) per kgm. of body weight. A tracheal cannula was always inserted, to be used in case artificial respiration became necessary.

Forty of the 80 cats used received the drug while their sciatic nerves were being stimulated, and 40 received it while at rest.

Half the cats in each group received the glycoside by intravenous injection, and the other half by intra-arterial injection. In the former case the drug was injected either into a jugular or into a saphenous vein; in the latter, it was injected into the aorta by means of a retrograde cannulation of the peripherally ligated inferior mesenteric artery. The solution was given from a sealed flask containing the glycoside, which was displaced by corresponding amounts of mercury passed intermittently or continuously from a graduated burette.

The speed of injection was so regulated that 0.2 mgm. of k-strophanthoside per kgm. of body weight was administered in 45 minutes. This was continued at a uniform rate until the death of the animal.

In order to avoid death from respiratory failure, artificial respiration was given as soon as any irregularities in the respiratory movements appeared.

In every case a continuous record of arterial pressure was taken by means of a mercury manometer connected to the carotid artery. A fall to zero of the arterial pressure was taken as the end point of the experiments.

In the experiments with muscular work, the sciatic nerves were cut below the point where the branches to the hamstrings leave the main trunk. These branches and the crural nerves were also cut. Shielded electrodes were placed on the distal segments of both sciatic nerves and maximal electric stimuli were applied at a frequency of five to six per second. The stimuli were condenser discharges with frequency controlled by thermionic tubes. In some experiments drills were inserted into the tibia to fix the limb, the Achilles' tendon was separated from its bony attachments and connected to a tension myograph and the muscular contractions were registered.

The k-strophanthoside<sup>1</sup> was used in a 1:50,000 solution in 0.85% NaCl, and freshly prepared for each experiment.

The hearts were weighed after removing the pericardium, the large vessels and the blood. In some experiments the muscles innervated by the stimulated portion of the sciatic nerves were weighed. The values thus obtained were compared with the body weights of the corresponding animals.

**RESULTS.** The average lethal dose per kilogram of body weight for the animals without muscular work was 0.250 mgm. for those injected intravenously and 0.244 mgm. for those injected intra-arterially. On the other hand, the average for the animals with muscular work was 0.270 mgm. for the intravenous series and 0.276 mgm. for the intra-arterial series. The values for the individual experiments, the standard deviations, and the standard errors are included in table 1.

The average time in which the animals without muscular work died was 56.4 min. and 54.4 min. respectively for those injected by vein and those injected by artery, and 60.45 min. and 62.75 min. for the corresponding groups with muscular work. The individual values are also included in table 1.

<sup>1</sup> The k-strophanthoside used in these experiments was kindly provided by Sandoz de Mexico, S. A.

TABLE 1

*Lethal doses of k-strophanthoside for cats, with or without muscular work, receiving the drug by intravenous or intra-arterial injection*

INTRAVENOUS INJECTION				INTRA-ARTERIAL INJECTION			
Experiment number	Body weight	Time	Lethal dose	Experiment number	Body weight	Time	Lethal dose
Animals without work							
	kgm.	min.	mgm. per kgm.		kgm.	min.	mgm. per kgm.
34	2.5	60	0.269	14	2.8	58	0.258
36	2.1	69	0.319	15	2.9	56	0.249
38	2.05	51	0.228	16	3.1	62	0.276
39	3.1	60	0.264	17	2.55	59	0.263
40	2.0	58	0.248	19	2.55	61	0.263
41	2.0	52	0.228	21	3.1	44	0.187
42	2.45	72	0.269	22	2.4	50	0.222
43	2.75	55	0.240	23	2.7	48	0.213
44	2.5	56	0.244	24	2.45	69	0.277
46	2.6	60	0.254	25	3.1	36	0.232
47	2.65	53	0.234	55	2.4	58	0.256
48	2.65	40	0.175	56	3.0	54	0.240
49	2.8	54	0.238	57	2.9	57	0.252
50	2.6	60	0.272	58	2.5	53	0.236
54	2.5	52	0.230	58b	2.83	49	0.208
87	2.8	72	0.321	100	2.7	55	0.241
88	2.85	41	0.181	101	2.5	61	0.268
89	2.4	45	0.265	102	2.45	62	0.277
90	2.4	56	0.248	103	2.65	51	0.229
105	2.85	62	0.272	104	2.55	55	0.241
Averages.....	2.53	56.4	0.2499		2.71	54.4	0.2441
Standard deviation.....			0.0351				0.0230
Standard error.....			0.0080				0.0052
Animals with work							
	kgm.	min.	mgm. per kgm.		kgm.	min.	mgm. per kgm.
68	2.7	66	0.293	26	3.0	60	0.266
69	2.8	40	0.177	27	2.5	68	0.302
70	2.5	51	0.224	29	2.6	70	0.310
71	2.5	65	0.286	30	2.25	60	0.271
72	2.9	63	0.280	31	2.3	73	0.322
73	2.7	70	0.311	52	2.85	74	0.308
74	2.45	56	0.250	53	2.85	61	0.272
75	2.7	81	0.360	61	2.5	61	0.269
76	2.15	64	0.284	62	2.35	65	0.286
77	2.15	65	0.287	64	2.9	58	0.258
78	3.0	67	0.296	64b	2.3	55	0.250
79	2.15	47	0.209	65	2.3	56	0.250
80	2.5	60	0.265	66	2.55	57	0.254
81	2.4	60	0.266	68b	3.0	55	0.244
82	2.4	58	0.258	91	2.7	55	0.222

TABLE 1—*Continued*

INTRAVENOUS INJECTION				INTRA ARTERIAL INJECTION			
Experiment number	Body weight	Time	Lethal dose	Experiment number	Body weight	Time	Lethal dose
Animals with work							
	kgm	min	mgm per kgm		kgm	min	mgm per kgm
85	2.8	58	0.257	92	2.25	54	0.240
86	2.9	59	0.262	95	2.85	64	0.280
106	2.55	64	0.281	97	2.65	86	0.377
107	2.85	56	0.250	98	2.9	44	0.194
108	2.05	69	0.304	99	2.4	79	0.343
Averages	2.56	60.5	0.2700		2.60	62.8	0.2759
Standard deviation			0.0379				0.0412
Standard error			0.0087				0.0094

In all the cases in which the muscular responses were registered, the amplitude of the contractions diminished gradually, and sometimes completely disappeared (fig. 1). The complete disappearance of muscular response was observed in the case of those animals that tolerated large doses of the glycoside. Muscles which were not being continuously stimulated were able to respond with normal amplitude even at the end of the intra arterial injection of the lethal dose (fig. 2A).

The reduction in the amplitude of the muscular responses was more rapid when the drug was injected by artery (fig. 1B) than when it was injected by vein (fig. 1A), judged by the fact that the contractions fell to 50% of the initial amplitude much sooner in the first case than in the second. However, complete inexcitability was reached at about the same time.

The average weight of the hearts, from 50 animals, was 10.198 grams (arithmetic mean) with standard deviation for the series of 2.03 grams. The average body weight in the same group of animals was 2.621 kgm with a standard deviation of 0.329 kgm. These figures represent a standard deviation of 19.9% for the heart weights, and 12.5% for the body weights. Complementing this marked difference in deviations, no correlation was found between the body weight and the weight of the heart. The coefficient of correlation was 0.65.

Vomiting, commonly provoked by digitalis principles in this type of experiment, was observed in 35% of the total number of cats. A larger percentage of animals vomited in the series injected intravenously (47.5%) than in the series injected intra arterially (25%). Moreover, in both series, the cats with continuous muscular activity vomited less frequently (27.5%) than those with no muscular work (45%).

**DISCUSSION** The method we have used to determine the lethal doses does not meet the specifications of official methods of bio assay for digitalis bodies. The differences in the methods employed preclude a comparison between the

average lethal dose, 0.247 mgm., for our series without muscular work and the figure, 0.127 mgm., obtained by Rothlin (10) with the same glycoside. MacDonald (11), using Dial as anesthetic, found an average lethal dose of 0.357 mgm. for Strophantin (B.P.), with 31 minutes as the average time of injection. Even though the time in which the drug is injected has a considerable influence on the magnitude of the lethal dose, this factor apparently does not contribute to the explanation of the difference between our values and those of Rothlin. The

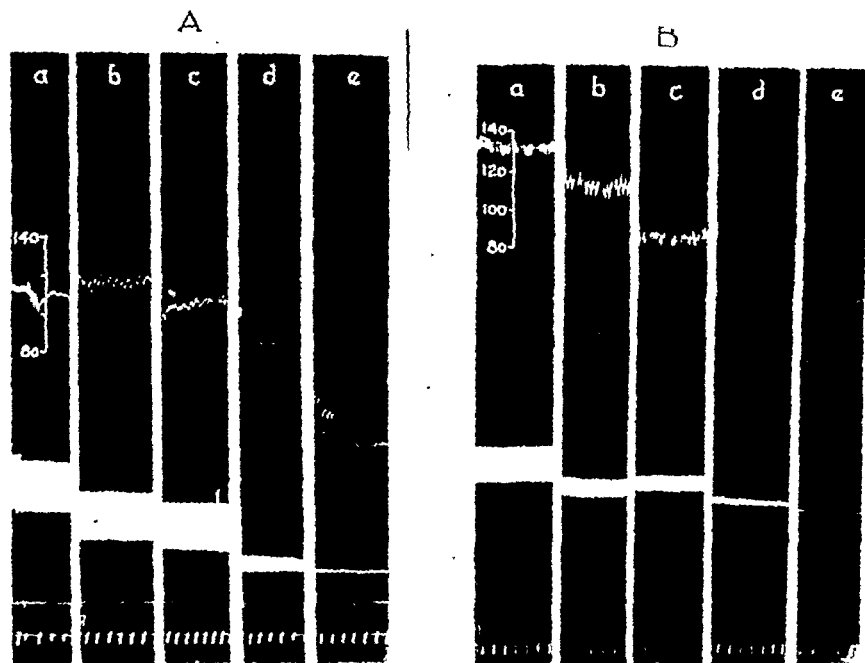


FIG. 1. PROGRESSIVE CHANGES IN THE AMPLITUDE OF MUSCULAR CONTRACTIONS DURING THE CONTINUOUS INJECTION OF K-STROPHANTHOSIDE

Cats under Dial anesthesia. Upper tracing: blood pressure. Calibration in mm. of mercury. Lower tracing: contractions of muscles upon indirect stimulation at a frequency of 5 per sec. intervals.

In both A and B the segments correspond to a, 10 min., b, 30 min., c, 50 min., d, 64 min. and e, 67 min. after the beginning of the injection of the k-strophanthoside.

A. Intravenous injection.

B. Intra-arterial injection.

dose becomes greater when the time of injection is reduced (12), and Rothlin's determinations were made using times shorter than ours.

Contrary to what was expected, the lethal doses obtained upon injection by vein and by artery were similar. It had been supposed that the intra-arterial injection of the drug would determine a greater storage by the skeletal muscles receiving the drug directly, resulting in a smaller remainder to be taken up by the heart and a correspondingly greater lethal dose. Such inferences were justified by the results of Weese (6) who used the method of complementary doses.

He found that the hearts of cats to which an initial dose of digitoxin had been injected into the aorta at the level of the left subclavian artery tolerated, in heart lung preparations, additional amounts of glycoside, which elevated the heart's total tolerance to 170% of that which would correspond to an intravenous infusion. Moreover, Lhotak (13) reported a greater lethal dose for digitoxin upon injection by cranial artery than upon injection by femoral vein. However,

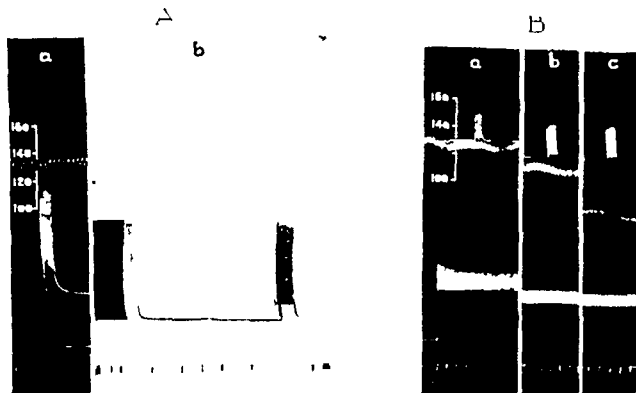


FIG. 2. ABSENCE OF FALL IN THE AMPLITUDE OF THE CONTRACTIONS OF MUSCLES RECEIVING K STROPHANTHOSIDE BY INTRA ARTERIAL INJECTION BUT NOT BEING CONTINUOUSLY STIMULATED (A), AND OF MUSCLES WHICH WERE BEING CONTINUOUSLY STIMULATED BUT WHICH RECEIVED NO K STROPHANTHOSIDE (B)

Cats under Dial anesthesia. Tracings of blood pressure calibrated in mm. of mercury, and of the contractions of stimulated indirectly at a frequency of 5 per sec.

A The muscles were stimulated continuously. s of time. a 1 min after the beginning of the injection of k strophanthoside. b 51 min later the first period of stimulation, 4 min before the animal's death, the second, immediately after cardiac arrest.

B The muscles of the left leg (lower tracing) were stimulated continuously, those of the right leg (upper tracing) were stimulated intermittently. a Beginning of stimulation. b 120 minutes later. c 5 minutes later. Between b and c the blood pressure was lowered by bleeding.

in our experiments equality of lethal doses for the animals injected by vein and for those injected by artery was observed in the animals without muscular work, as well as in those subjected to it. The statistical significance of the difference between the doses for the animals injected by vein and those for the animals injected by artery was 0.013, a value so low as to imply practical equality for the two series.

The equality in the doses by either route of administration, in the case of the animals without muscular work, could be interpreted as indicative of a negligible storage of k strophanthoside by striated muscle. However, this interpretation

cannot be applied to the series of animals with muscular work, for the increase in the lethal dose may be interpreted as evidence of the storage of an appreciably greater amount of k-strophanthoside by the muscles activated. On the other hand, whatever the amount of glycoside taken up by the muscles may be, if the failure of the heart is secondary to a slow or fast, but similar, saturation of those muscles, the lethal dose should be the same independently of the route of administration.

In the experiments with muscular work, the amplitude of the muscular responses fell much more rapidly in the animals injected by artery than in those injected by vein. This observation is in apparent contradiction with the equality of lethal doses found when one or the other of the routes of administration was used. However, as eventually muscular contractility disappeared completely or almost completely, it can be assumed that sooner or later the muscles reached a similar degree of saturation.

TABLE 2

*Analysis of the influence of the route of injection and of muscular activity on the lethal dose of k-strophanthoside for cats*

	ROUTE OF INJECTION		MUSCULAR ACTIVITY	
	Vein	Artery	Without	With
Average lethal dose	0.2599	0.2600	0.2470	0.2730
Standard deviation.	0.03626	0.03360	0.02965	0.03956
Standard error	0.00573	0.00531	0.00468	0.00625
Significance of difference	0.013		3.329	

The failure of muscular response in the observations here described, and the inexcitability of directly stimulated muscles already reported (9), must be due to an action of the digitalis bodies, for, in normal conditions, the muscles in question continue to respond for many hours (fig. 2B) to the frequency of stimulation employed (14).

If the inexcitability of striated muscle means saturation with glycoside, as the equality of lethal doses for the arterial and the venous routes suggests, the capacity of the muscle for storage of the drug is probably independent of the concentration in which it is received. This property would be similar to that described for cardiac muscle by Weese (3). In both cases the concentration of the administered glycoside would only determine the rate of storage, but not the total amount taken up.

In view of the fact that no significant statistical difference was found between the lethal dose determined by intra-arterial injection and that determined by intravenous injection, the total number of animals with no muscular work can be compared directly with the total number with muscular activity. Thus considered, the average lethal dose was 0.273 mgm.  $\pm$  0.00625 (S.E.) for the animals with work, and 0.247 mgm.  $\pm$  0.00468 for those without. The statistical

significance of the difference between these series, by the "t" test, was 3.32, which means that the lethal doses for the animals with work were significantly higher than those for the animals without muscular activity.

The increase in the work of the heart which could result from muscular exercise could be expected to make the values for the cats in activity smaller; in our observations they were greater. Apart from this, Farah (15), using heart lung preparations, found that the lethal dose for g-strophanthin is independent of the amount of work done by the heart.

The difference between the average lethal dose for the animals with and for those without muscular work, 0.026 mgm., may be taken as the amount stored by the muscles as a result of their activity. That this amount would represent a not unimportant storage is evidenced by the fact that the muscles activated were only 2.8% of the body weight. No statement of the mechanism of such possible increased storage can be made. Changes in blood supply and in permeability, brought about by exercise, may be of importance.

These results could have a definite bearing on the extraordinary ability of the heart to take up digitalis glycosides, given the fact that the heart has an abundant blood supply and is constantly in activity. The results of Farah's work (12) in no way invalidate this assumption, for he compared only the storage by the heart while in different grades of activity, but did not determine the storage while at rest.

No figures relating lethal dose and heart weight are given because the correlation found was not significant. The coefficients of correlation for the series with work and that without were 0.64 and 0.72 respectively. This seems to agree with the fact that the heart stores only 10% of administered digitalis bodies (2), and with the absence of correlation between the weight of the heart and that of the body. These results are not in agreement with Chen's report (16) of close correlation between heart and body weights in cats.

#### SUMMARY

The lethal dose of k-strophanthoside was determined in 40 cats subjected to muscular work by the continuous stimulation of both sciatic nerves. The average lethal dose for these animals was significantly greater than that determined under identical conditions in 40 cats without muscular activity.

Half of the animals in each series received the glycoside by intravenous injection, and the other half by intra-aortic injection at the level of the inferior mesenteric artery. The lethal doses were the same for the two routes.

The amplitude of muscular contraction fell more rapidly in the animals injected by artery than in those injected by vein. However, in both cases the inexcitability of the muscles was complete or nearly complete before cardiac arrest.

These results may be interpreted as indicating that the work of striated muscle considerably increases its capacity for storage of k-strophanthoside. The possible influence of cardiac activity on the selective storage of digitalis bodies by the heart is discussed.



## REFERENCES

1. HATCHER, R. A., AND EGGLESTON, G.: THIS JOURNAL, 12: 405, 1919.
2. ROTHLIN, E.: Schweiz. med. Wchnschr., 74: 217, 1944.
3. WEESE, H.: Arch. f. exper. Path. u. Pharmacol., 135: 228, 1928.
4. SCHLIOMENSUM, B.: Arch. f. exper. Path. u. Pharmacol., 63: 294, 1910.
5. FISCHER, H.: Arch. f. exper. Path. u. Pharmacol., 130: 111, 1928.
6. WEESE, H.: Arch. f. exper. Path. u. Pharmacol., 141: 329, 1929.
7. DEL POZO, E. C.: Bol. Lab. Est. Med. Biol., 2: 93, 1943.
8. HATCHER, R. A., AND BRODY, J. G.: Am. J. Pharm., 82: 360, 1910.
9. DEL POZO, E. C.: Gac. méd. de México, 74: 400, 1944.
10. ROTHLIN, E.: Pharm. Acta Helvet., 22: 418, 1947.
11. MACDONALD, A. D.: Quart. J. Pharm. & Pharmacol., 7: 182, 1934.
12. FARAH, A.: THIS JOURNAL, 86: 101, 1946.
13. LHOTÁK VON LHOTA, C.: Biochem. Ztschr., 48: 144, 1913.
14. DEL POZO, E. C.: Am. J. Physiol., 135: 763, 1942.
15. FARAH, A.: THIS JOURNAL, 87: 364, 1946.
16. CHEN, K. K., BLISS, C. I., AND ROBBINS, E.: THIS JOURNAL, 74: 223, 1942.

# THE FAILURE OF 2,3 DITHIOPROPANOL (BAL) TO AFFECT ACUTE, SYSTEMIC URANIUM POISONING<sup>1</sup>

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Investigations of the pharmacology and toxicology of uranium compounds (1) have indicated that hexavalent uranium is not a very effective enzyme poison at physiological pH and has little affinity for sulfhydryl groups. Tetravalent uranium, however, combines with sulfhydryl groups to some extent. Therefore, BAL may be expected to reduce the toxicity of parenterally administered uranyl compounds only by forming a somewhat stable, non-toxic  $U_4$ -BAL complex following reduction of hexavalent uranium *in vivo*. Because of the effectiveness of BAL in the treatment of heavy metal poisoning (2-4) and the contra-indication of BAL in experimental, systemic cadmium poisoning (5), it seemed imperative to test BAL against acute, experimental uranium poisoning. The results indicated that BAL exerted no significant effect on the course of acute uranium poisoning following parenteral administration of uranyl or uranous compounds.

Since these experiments were completed<sup>2</sup>, a report has appeared (6) stating that BAL increases the toxic effects of uranium administered to dogs.

**EXPERIMENTAL.** Groups of twenty adult, male, Wistar rats, weighing from 250-350 gm. each were observed and weighed on several days prior to injection. Following injection, all animals were weighed on alternate days and observed for at least one month, a period judged adequate since most deaths occurred within ten days and none later than seventeen days. Mortality accompanied by gross kidney pathology was taken as the ultimate criterion of effectiveness or lack of protection of BAL against uranium poisoning. It is understood that most survivors sustained renal injury to a varying degree, but mortality appeared to be a convenient quantitative measure of the functional damage produced by uranium.

All solutions of BAL were prepared immediately prior to intramuscular injection in either physiological saline or propylene glycol. Since propylene glycol was found to be irritating, saline solutions were used exclusively in later experiments.

A pure sample of BAL was obtained from the Chemical Warfare Service (G-40 Special, Lot No. E11-D). The toxicity of this preparation for rats was found to be approximately the same as that determined by both the British (7) and American (8) investigators for earlier preparations ( $LD_{50}$  equal to 110.0 mgm./kgm. subcutaneously, and 105 mgm./kgm. intramuscularly).

*The Efficacy of BAL in the Treatment of Systemic  $HgCl_2$  Intoxication in Rats.* The rat seemed to be the animal of choice because of the large amount of avail-

<sup>1</sup> This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

<sup>2</sup> The content of this paper has been taken entirely from University of Rochester Atomic Energy Project Report No. M-1980, May 1947.

Since the —SH groups of BAL are readily available for combination with sulfhydryl-reactive metals such as arsenic and mercury, it is probable from the mortality data in this study that no significant *in vivo* reaction occurs between BAL and uranium. This is in keeping with the *in vitro* enzyme studies (1a) which demonstrated that  $U_6$  has practically no effect on "sulfhydryl" enzymes.

Although  $UCl_4$  possesses some affinity for —SH groups (1a) a host of other substances in the body are also effective in complexing  $U_4$ . The fact that the toxicity of  $U_4$  remained unaltered in the face of BAL therapy would indicate that —SH compounds were unable to compete successfully for  $U_4$  *in vivo*.

#### SUMMARY

1. Albino rats were systemically poisoned with 3.0 mgm./kgm. of  $HgCl_2$  I.P., and successfully treated with BAL as late as 30 minutes after the administration of Hg.

2. Studies were made of the effect of BAL on the toxic effects of three uranium compounds ( $UO_2(NO_3)_2 \cdot 6H_2O$ ,  $UO_2F_2$  and  $UCl_4$ ) parenterally administered to albino rats with negative results.

3. The negative results were taken as confirmatory evidence for the view that uranium exerts its toxic effects primarily by some other means than a combination with —SH groups.

#### REFERENCES

1. N.N.E.S., Div. VI, Vol. IA, The Pharmacology and Toxicology of Uranium Compounds, McGraw-Hill Publishing Co., (in press). a. Chapter XIII, Dounce, A. L. and Lan, T. H. b. Chapter XV, Dounce, A. L. c. Chapter VI, Haven, F. L. and Hodge, H. C. d. Chapter I, Dounce, A. L. and Flagg, J. F.
2. CARLETON, A. B., PETERS, R. A., STOCKEN, L. A., THOMPSON, R. H. S., AND WILLIAMS, D. I.: J. Clin. Investigat. 25: 497, 1946.
3. LONGCOPE, W. T., LUETSCHER, J. A., WINTROBE, M. M., AND JAGER, V.: J. Clin. Investigat., 25: 528, 1946.
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5. GILMAN, A., PHILIPS, F. S., ALLEN, R. P., AND KOELLE, E. S.: THIS JOURNAL, 87: 85, 1946.
6. McNIDER, W. DEB., TROTT, JR., J. C., AND BRUCE, M. D.: THIS JOURNAL, 94: 262, 1948.
7. LONGCOPE, W. T., AND LUETSCHER, J. A.: J. Clin. Investigat., 25: 557, 1946.
8. GILMAN, A., PHILIPS, F. S., ALLEN, R. P., AND KOELLE, E. S.: THIS JOURNAL, 87: 85, 1946.
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10. ROSENTHAL, S. M.: Pub. Health Reports, 48: 1543, 1933.
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**EXPERIMENTAL.** Groups of twenty adult, male, Wistar rats, weighing from 250-350 gm. each were observed and weighed on several days prior to injection. Following injection, all animals were weighed on alternate days and observed for at least one month, a period judged adequate since most deaths occurred within ten days and none later than seventeen days. Mortality accompanied by gross kidney pathology was taken as the ultimate criterion of effectiveness or lack of protection of BAL against uranium poisoning. It is understood that most survivors sustained renal injury to a varying degree, but mortality appeared to be a convenient quantitative measure of the functional damage produced by uranium.

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able data (1) on the toxicology of uranium. There was, however, little information available as to the response of the albino rat to BAL therapy in other metal intoxication. To compare the rat with other species (9), a preliminary experiment was performed to determine the efficacy of BAL against mercury poisoning in this animal.

HgCl<sub>2</sub> was administered intraperitoneally<sup>3</sup>, 3.0 mgm./kgm. as a 3.0 per cent solution in saline to 75 rats. One group of 35 served as controls; a second group of 20 received BAL as in Schedule A<sup>4</sup> and a third group of 20 received BAL as in Schedule B<sup>4</sup>. BAL was given as a 2 per cent solution in saline.

The control group showed 89 per cent mortality, the majority of deaths occurring 4 and 5 days after injection, with a maximum, average weight loss of 20 per cent. The group receiving BAL following Hg showed but 5 per cent mortality with a maximum average weight loss of only 4.2 per cent. The group treated with BAL before and after Hg, showed no mortality and an average, maximum weight loss of 4.0 per cent. Animals which succumbed were autopsied, and only the kidneys were grossly abnormal.

These data proved the rat to be responsive to BAL therapy in acute mercury intoxication. The use of rats to assay the efficacy of BAL in combating uranium poisoning appeared justified.

*The Efficacy of BAL in the Treatment of Systemic Uranium Intoxication in Rats.* BAL was assayed against three representative uranium compounds: hexavalent uranyl nitrate (UO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O), uranyl fluoride (UO<sub>2</sub>F<sub>2</sub>), and tetravalent uranium tetrachloride (UCl<sub>4</sub>).

Sixty rats were given 3.0 mgm. U/kgm., intraperitoneally as a 0.63 per cent aqueous solution of uranyl nitrate hexahydrate. Though this quantity had been shown to give 75 per cent mortality (1c), slightly more than 90 per cent of the animals succumbed in this experiment, presumably because of their greater age (1c). Sixty rats were given intraperitoneally 2.5 mgm. U/kgm. as a 0.324 per cent aqueous solution of UO<sub>2</sub>F<sub>2</sub>. From available data (1c), this amount was judged to be lethal to the majority of the rats so injected.

The animals injected with either uranium compound were divided into three groups of twenty each. One group served as controls, one received BAL according to Schedule A<sup>4</sup> and one was given BAL as in Schedule B<sup>4</sup>. BAL as a 1.24 per cent solution in propylene glycol was injected intramuscularly. Though the amount of BAL given was but slightly more than half that given in the mercury experiment, this dose appears more than adequate (9) to reduce mortality where BAL at all effective.

<sup>3</sup> In a preliminary study, intravenous administration resulted in 24 hr. deaths from pulmonary edema and pleural effusion. This finding is in agreement with studies utilizing dogs (9, 10). Accordingly, all animals were given HgCl<sub>2</sub> intraperitoneally.

<sup>4</sup> BAL administered on Schedule A was given in three, equally divided, intramuscular doses 5 min. before, and 1 and 3 hrs. after the injection of the toxic metal; on Schedule B in three, equally divided, intramuscular doses,  $\frac{1}{2}$ , 1 $\frac{1}{2}$ , and 3 $\frac{1}{2}$  hours after the metal-injection. The volume injected was always 0.1 cc./100gm. rat/dose. It was expected that BAL, if effective, might be most beneficial given before uranium administration since this element leaves the blood stream very rapidly (11).

The results are given in table I. BAL exerted no significant effect on the course of systemic intoxication from either uranyl nitrate or fluoride.

As stated above, BAL was expected to exert a beneficial effect in uranium poisoning only if a reduction of hexavalent uranium to the tetravalent state took place *in vivo*. Actually, this reduction appeared unlikely from the results of studies of the redoxpotential of the  $U_6:U_4$  system. It was of theoretical interest, therefore, to assay BAL against uranous chloride.

Unlike hexavalent uranium,  $U_4$  has been shown to complex *strongly* with protein under physiological conditions (1a). Since the absorption of  $U_4$  from the peritoneal cavity was problematical, uranous chloride was injected intravenously. Uranous chloride is very acid and autoxidizes on standing; therefore, the dry material was dissolved in 1 M sodium acetate containing a trace of sodium hydro-sulfite. The solution injected contained approximately 5.0 mgm.  $UCl_4$ /cc.

TABLE I  
*The failure of BAL to affect mortality from uranium poisoning*

TOXIC AGENT	MORTALITY			TOTAL BAL DOSAGE
	Controls	BAL (Schedule A)	BAL (Schedule B)	
	%	%	%	mgm /kgm
$UO_2(NO_3)_2$	95	100	85	37.2
$UO_2F_2$	84.2*	85	85	37.2
$UCl_4$	55	45	—	60
$UCl_4$	37*	—	40	60

\* One animal succumbed accidentally and was not included in the experiment.

A preliminary study indicated that 5.1 mgm./kgm. of  $UCl_4$  was lethal to about 50 per cent of the rats so injected. Accordingly, forty rats under light ether anesthesia were injected with approximately 5 mgm./kgm. of  $UCl_4$  via the femoral vein. Twenty rats served as controls; twenty received BAL as a 2 per cent solution in saline according to Schedule A<sup>4</sup>. The results are presented in table I. The slight reduction in mortality in the BAL treated group is of very doubtful significance and certainly does not warrant further investigation.

A second experiment was performed in which BAL was given as in Schedule B<sup>4</sup>; all other details were as before. In this case, BAL afforded no protection as indicated by the data presented in table I.

DISCUSSION. It is evident from the above data that BAL was completely ineffective in modifying the course of systemic uranium intoxication in albino rats. As measured by mortality and weight loss, there was no amelioration or enhancement of toxic effects caused by the administration of three uranium compounds to rats when BAL therapy was initiated either before or after uranium injection. This is not in complete agreement with McNider and co-workers (6) who concluded that BAL actually "enhances the toxicity" in dogs. This may be due to a species difference. On the other hand, data were presented on only five of the comparatively small number of animals studied (6).

Since the —SH groups of BAL are readily available for combination with sulfhydryl-reactive metals such as arsenic and mercury, it is probable from the mortality data in this study that no significant *in vivo* reaction occurs between BAL and uranium. This is in keeping with the *in vitro* enzyme studies (1a) which demonstrated that  $U_6$  has practically no effect on "sulfhydryl" enzymes.

Although  $UCl_4$  possesses some affinity for —SH groups (1a) a host of other substances in the body are also effective in complexing  $U_4$ . The fact that the toxicity of  $U_4$  remained unaltered in the face of BAL therapy would indicate that —SH compounds were unable to compete successfully for  $U_4$  *in vivo*.

#### SUMMARY

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1. N.N.E.S., Div. VI, Vol. IA, The Pharmacology and Toxicology of Uranium Compounds, McGraw-Hill Publishing Co., (in press). a. Chapter XIII, Dounce, A. L. and Lan, T. H. b. Chapter XV, Dounce, A. L. c. Chapter VI, Haven, F. L. and Hodge, H. C. d. Chapter I, Dounce, A. L. and Flagg, J. F.
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3. LONGCOPE, W. T., LUETSCHER, J. A., WINTROBE, M. M., AND JAGER, V.: J. Clin. Investigat., 25: 528, 1946.
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7. LONGCOPE, W. T., AND LUETSCHER, J. A.: J. Clin. Investigat., 25: 557, 1946.
8. GILMAN, A., PHILIPS, F. S., ALLEN, R. P., AND KOELLE, E. S.: THIS JOURNAL, 87: 85, 1946.
9. GILMAN, A., ALLEN, R. P., PHILIPS, F. S., AND ST. JOHN, E.: J. Clin. Investigat., 25: 549, 1946.
10. ROSENTHAL, S. M.: Pub. Health Reports, 48: 1543, 1933.
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# A SIMPLIFIED METHOD OF EVALUATING DOSE-EFFECT EXPERIMENTS

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The increased emphasis on quantitative biological studies in recent years has resulted in the widespread use of statistical methods for evaluating biological data. Much of this data is of the all-or-none type and, consequently, it is necessary to solve a dose-per cent effect curve. By converting doses to logarithms and per cent effects to probits (1), logits (2), or angles (3), a straight line may be fitted by the method of weighted least squares. From the viewpoint of many biologists, such procedures are not pleasant to contemplate because the data must be converted to units which are meaningless to many and the calculations are difficult, tedious and often quite incomprehensible. It is not surprising therefore that there is widespread use of a variety of approximate methods for solving dose-per cent effect curves. It may be argued that such methods are undesirable because they do not make use of all of the information contained in the data, and are therefore inefficient in a statistical sense. On the other hand, the computations necessary in using efficient methods are often so time-consuming and laborious that the busy experimenter is deterred from using them, and thus loses the advantage of a statistical evaluation of his results. An examination of the various approximate methods, which have been proposed for the solution of dose-effect experiments of the all-or-none type, leads to the conclusion that none of them are entirely satisfactory in combining ease of computation with efficiency and accuracy. In order to appreciate this fact, it is helpful to list the essentials of a satisfactory approximate method for the solution of dose-effect experiments.

(1) The method should give not only the  $ED_{50}$  and slope of the curve, but also their confidence limits.

(2) The method should use the data in original units throughout.

(3) Zero and 100 per cent effects should be used effectively.

(4) The method should make it possible to carry out the necessary calculations within 10-15 minutes without a calculating machine, and without resort to logarithms.

(5) The method should recognize heterogeneity when present and give corrected confidence limits in such cases.

(6) The method should facilitate both the comparison of the two curves for parallelism and the computation of relative potency with its confidence limits.

(7) The method should not unduly sacrifice accuracy in favor of simplicity and speed.

The various approximate methods for solving dose-per cent effect curves fail



in varying degrees to meet these requirements. The double integration method (4-7) which uses data in original units and the methods of averages (8-10) which require logarithms provide an estimate of the  $ED_{50}$ . With a restricted experimental design, the confidence limits of the  $ED_{50}$  can be obtained by the methods of averages, with varying amounts of calculation.

Of the methods which may be considered to approximate that of Bliss (1), one, using the data in original units gives only the confidence limits of the  $ED_{50}$  (11); a second, using logarithms gives, in addition to the above, the slope constant, but not its limits (12); and a third, using logarithms and probits gives both parameters and their confidence limits (13). None of the approximate methods use 0 or 100 per cent observations to best effect, or recognize heterogeneity, if present.

The method of Litchfield and Fertig (13), which gives confidence limits of both parameters, appeared to offer the best starting point for developing a revised method which would approach the ideal requirements mentioned above.

In order to revise the above method to conform to the aims listed, three distinct types of problem were involved. The first of these was the problem of using percentages and arithmetic values in a way exactly equivalent to the use of logarithms and probits. Logarithmic-probability paper permits plotting the data in original units but leaves the problem of converting log-probit equations to their arithmetic equivalent.

The result of addition and subtraction of logarithms can be obtained easily by multiplication and division of the numbers themselves. Similarly, the result of multiplying or dividing a logarithm by an arithmetic value can be represented by raising the number corresponding to the logarithm to a power equal to the arithmetic value (or by taking the root in the case of division). Such a calculation cannot be made, however, without recourse to logarithms or to the use of log-log slide rule. Consequently, the second major problem arises, the need for eliminating calculations which require logarithms. In this particular case, a nomograph was constructed for obtaining fractional powers and roots of numbers coming within the scope of the method.

A further complication arises in the case of the product or quotient of two logarithms since this operation cannot be represented at all as a purely arithmetic process. In the two such cases which arise in the revised method, nomographs were constructed to permit solution without recourse to logarithms or a log-log slide rule.

By means of two of the above mentioned nomographs, a simple arithmetic solution of a dose-effect curve can be obtained which is equivalent to the solution by the original method using logarithms and probits. The two solutions are numerically equal but the revised method is more rapid and permits using the data in its original form.

The third type of problem in the revision consisted of finding the means for adding to the method a simple test for heterogeneity or goodness of fit of the line, together with the correction of confidence limits for heterogeneity; a means for effectively using 0 and 100 per cent effects in fitting the line to the data;

and lastly, the means for approximating the confidence limits of doses other than the Median Effective Dose.

The complete method including the necessary tables and nomographs is presented below, together with several examples illustrating its application.

**METHOD.** The following symbols are used in this method:

$K$  = the number of doses plotted

$n = K - 2$  = degrees of freedom for  $(\text{Chi})^2$

$t$  = value of "Student's"  $t$  for  $p = .05$

$\text{ED}_{50}$  = Median Effective Dose

$S$  = Slope function

$f_{\text{ED}_{50}}$  and  $f_S$  = factors for  $\text{ED}_{50}$  and  $S$ , respectively

$N'$  = total number of animals used between 16 and 84 per cent expected effects

$R$  = the ratio of largest to smallest dose plotted

$A$  = a value derived from  $S$  and  $R$

$S.R.$  and  $P.R.$  = Slope function Ratio and Potency Ratio

$f_{S.R.}$  and  $f_{P.R.}$  = factors for  $S.R.$  and  $P.R.$ , respectively

Unless otherwise indicated all ratios are taken as: larger/smaller value.

**PROCEDURE.** *A. The data and graph.* 1. List the actual doses used, the number reacting/number tested, and the per cent effects. Do not list more than two consecutive 100 per cent effects at the upper end or more than two consecutive 0 per cent effects at the lower end of the curve.

2. Plot doses against per cent effect on logarithmic-probability paper (No. 312S, Codex Book Co., Inc., Norwood, Mass.) leaving space for but omitting any 0 or 100 per cent effects.

With a transparent straight edge or triangle fit a temporary straight line through the points, particularly those in the region of 40 to 60 per cent effect.

*B. Plotting 0 or 100 per cent effects.* 1. Read and list the expected per cent effect, as indicated by the line drawn, for each dose tested. If the expected value for any dose is less than .01 or greater than 99.99 delete such doses and effects from the list.

2. Using the expected effect record and plot from table 1 a corrected value for each 0 or 100 per cent effect which is listed. Inspect the fit of the line to the completely plotted data. If it is obviously unsatisfactory refit the line and repeat the preceding two steps to obtain a new set of expected and corrected values.

When the line appears to fit satisfactorily, as is almost always the case with the first line, proceed to the  $(\text{Chi})^2$  test.

*C. The  $(\text{Chi})^2$  test.* 1. List the difference between each observed (or corrected) effect and the corresponding expected effect.

2. Using each difference and the corresponding expected effect read and list the contributions to  $(\text{Chi})^2$  from Nomograph No. 1. (A straight edge connecting a value on the expected per cent scale with a value on the difference scale, will indicate at the point of intersection of the  $(\text{Chi})^2$  scale, the contribution to  $(\text{Chi})^2$ .)

3. Total the contributions to  $(\text{Chi})^2$  and multiply by the average number of animals per dose, i.e., the total number of animals/ $K$ , the number of doses. This is the  $(\text{Chi})^2$  of the line. The degrees of freedom are two less than the number of doses plotted, i.e.,  $n = K - 2$ .

4. If the  $(\text{Chi})^2$  of the line is less than the value of  $(\text{Chi})^2$  given in table 2 for  $n$  degrees of freedom, the data are not significantly heterogeneous, i.e., the line is a good fit. If the  $(\text{Chi})^2$  of the curve exceeds the value of  $(\text{Chi})^2$  given in table 2, the data are significantly heterogeneous and the line is not a good fit. (If the  $(\text{Chi})^2$  of the line cannot be reduced below the permissible  $(\text{Chi})^2$  by refitting the line, the value of  $t$  in table 2 for  $n$  degrees of freedom should be noted.)

*D. The  $\text{ED}_{16}$  and  $f_{\text{ED}_{16}}$ .* 1. Read from the line on the graph the dose for 16, 50, and 84 per cent effects ( $\text{ED}_{16}$ ,  $\text{ED}_{50}$  and  $\text{ED}_{84}$ ).

2. Calculate the slope function,  $S$ , as:

$$S = \frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$$

3. Obtain from the data tabulation,  $N'$ , the total number of animals tested at those doses whose *expected effects* were between 16 and 84 per cent.

4. Calculate the exponent in the expression:

$$f_{ED_{50}} = S^{2.77/\sqrt{N'}} = S^{\text{exponent}}$$

To carry out this step, obtain first the  $\sqrt{N'}$  from a square root table, or with a slide rule, or by means of Nomograph No. 2. Then solve  $2.77/\sqrt{N'} = \text{exponent}$ . Next, using this exponent and the value of  $S$ , read the  $f_{ED_{50}}$  on the center scale of Nomograph No. 2 by laying a straight edge across the correct scale values.

5. Calculate the confidence limits of the  $ED_{50}$  as:

$$\left. \begin{array}{l} ED_{50} \times f_{ED_{50}} = \text{upper} \\ ED_{50}/f_{ED_{50}} = \text{lower} \end{array} \right\} \text{limit for 19/20 probability.}$$

E.  $S$  and  $f_S$

1. Calculate the dosage range as a ratio, as follows:

$$R = \text{largest/smallest dose plotted}$$

2. Using this value of  $R$  and that of  $S$  (from step D2), read the value designated as  $A$  from Nomograph No. 3 by laying a straight edge across the correct scale values.

3. Solve for the exponent in the following expression, using  $K$  from step C3 and  $\sqrt{N'}$  from step D4.

$$f_S = A^{10(K-1)/K\sqrt{N'}} = A^{\text{exponent}}$$

Then with this exponent and the value of  $A$ , read  $f_S$  from Nomograph No. 2.

4. Calculate the confidence limits of  $S$  as:

$$\left. \begin{array}{l} S \times f_S = \text{upper} \\ S/f_S = \text{lower} \end{array} \right\} \text{limit for 19/20 probability.}$$

F. *The factors for significantly heterogeneous data.* When the  $(\text{Chi})^2$  test indicates significant heterogeneity the value of  $t$  from table 2 is noted and the formulas below are used for the factors instead of those in steps D4 and E3. The procedure for solution consists of solving first for the values of the exponent and then with the value of  $S$  from step D2 and that of  $A$  from step E2, the factors are read from Nomograph No. 2.

$$1. f_{ED_{50}} = S^{1.4t\sqrt{(\text{Chi})^2/nN'}} = S^{\text{exponent}}$$

$$2. f_S = A^{[5.1t(K-1)\sqrt{(\text{Chi})^2/nN'}]/K} = A^{\text{exponent}}$$

G. *The test for parallelism of two lines and the estimate of relative potency.* The following values which represent the parameters and factors of a dose-per cent effect line are to be compared to a similar set of values for a second line:

$$\left. \begin{array}{l} ED_{50} \text{ and } f_{ED_{50}} \\ S \text{ and } f_S \end{array} \right\} \text{for each line.}$$

1. The test for parallelism: the slope function ratio, S.R.

(a) Calculate:  $S.R. = S_1/S_2$  where  $S_1$  is the larger value.

(b) Using  $f_{S_1}$  and  $f_{S_2}$  read  $f_{S.R.}$  from the center scale of Nomograph No. 4 by laying a straight edge across the correct scale values.

(c) If the value of S.R. exceeds the value of  $f_{S.R.}$  the curves deviate significantly (19/20 probability) from parallelism. If S.R. is less than  $f_{S.R.}$  the curves may be considered parallel within experimental error and the potency ratio may then be computed as follows:

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determine the potency ratio of the two drugs. The solution of the dose effect curve of Pyribenzamine illustrates the combined work sheet and graph.

The parameters and factors of the two dose effect curves are summarized below.

	Tagathen	Pyribenzamine
$ED_{50}$	0.18 mgm /kgm	0.60 mgm /kgm.
$f_{ED_{50}}$	1.72	1.60
S	2.20	2.34
$f_S$	1.60	1.57

The curves are tested for parallelism and the potency ratio obtained as follows:

G1 The slope ratio,  $SR = S_1/S_2 = 2.34/2.20 = 1.06$

$f_{SR} = 1.92$  (from Nomograph No. 4, using the two  $f_S$  values)

$SR$  of 1.06 is less than  $f_{SR}$  of 1.92, therefore, the deviation from parallelism is not significant.

G2 The potency ratio,  $PR = ED_{501}/ED_{502} = 0.60/0.18 = 3.3$

$f_{PR} = 2.05$  (from Nomograph No. 4, using the two  $f_{ED_{50}}$  values)

$PR$  of 3.3 exceeds  $f_{PR}$  of 2.05, therefore, the two drugs differ significantly in potency.

G3 Confidence limits of  $SR$  and  $PR$

$SR \times f_{SR} = 1.06 \times 1.92 = 2.03$

$SR / f_{SR} = 1.06 / 1.92 = 0.55$

The slope ratio,  $SR$ , and 19/20 confidence limits: 1.06 (0.55 to 2.03)

$PR \times f_{PR} = 3.3 \times 2.05 = 6.75$

$PR / f_{PR} = 3.3 / 2.05 = 1.60$

The potency ratio,  $PR$ , and 19/20 confidence limits: 3.3 (1.6 to 6.8). Tagathen was significantly more active than Pyribenzamine and for confidence limits of 19/20, its relative activity lies between 1.6 and 6.8 times that of Pyribenzamine.

Occasionally the experimenter is interested in a dose other than the  $ED_{50}$ , for example, he may wish to know the  $ED_{90}$  and its confidence limits. The dose for any desired per cent effect,  $Y$ , can be read from the graph. The 19/20 confidence limits of this dose,  $ED_Y$ , can be approximated by increasing the value of  $f_{ED_{50}}$  by an amount determined by the value of  $f_S$  and  $\lambda$ , (the deviation in standard deviation units, of  $Y$  from 50 per cent). Values of  $\lambda$  for common values of  $Y$  are given in table 3.

The procedure for obtaining the 19/20 confidence limits of  $ED_Y$  is as follows:

1 Obtain  $(f_S)^X$  using Nomograph No. 2 to raise the base,  $f_S$  (from step E3) to the exponent  $\lambda$  (from table 3).

2 Obtain  $f_{ED_Y}$  from center scale of Nomograph No. 4, using the value obtained for  $(f_S)^X$  and the value of  $f_{ED_{50}}$  (from step D4). If the scale limits of the nomograph are exceeded, the confidence limits are likely to be so wide that the  $ED_Y$  value is rather meaningless. The confidence limits are obtained in the usual way using the  $f_{ED_Y}$ .

As an example of this procedure applied to the Tagathen line, the confidence limits of  $ED_{90} = 0.48$  mgm /kgm are obtained as follows:

1  $(f_S)^X = (1.60)^{1.8} = 1.85$  (from Nomograph No. 2 and table 3)

2  $f_{ED_{90}} = 2.25$  (from Nomograph No. 4, using  $(f_S)^X = 1.85$  and  $f_{ED_{50}} = 1.72$ )

$ED_{90}$  and 19/20 confidence limits = 0.48 (0.21 to 1.05) mgm /kgm

**DISCUSSION** The method presented fulfills, for the most part, the aims of a satisfactory approximate method. At the risk of making the method appear rather complex the instructions have been made as complete as possible. We have found that these instructions in the hands of an inexperienced person permit a complete solution of data, such as that in fig. 1, to be obtained in 20 to 30 minutes. An experienced person on the other hand requires less than half

this time. Although the accuracy of this method has not been examined, it cannot be less than that of the Litchfield-Fertig method which has been shown

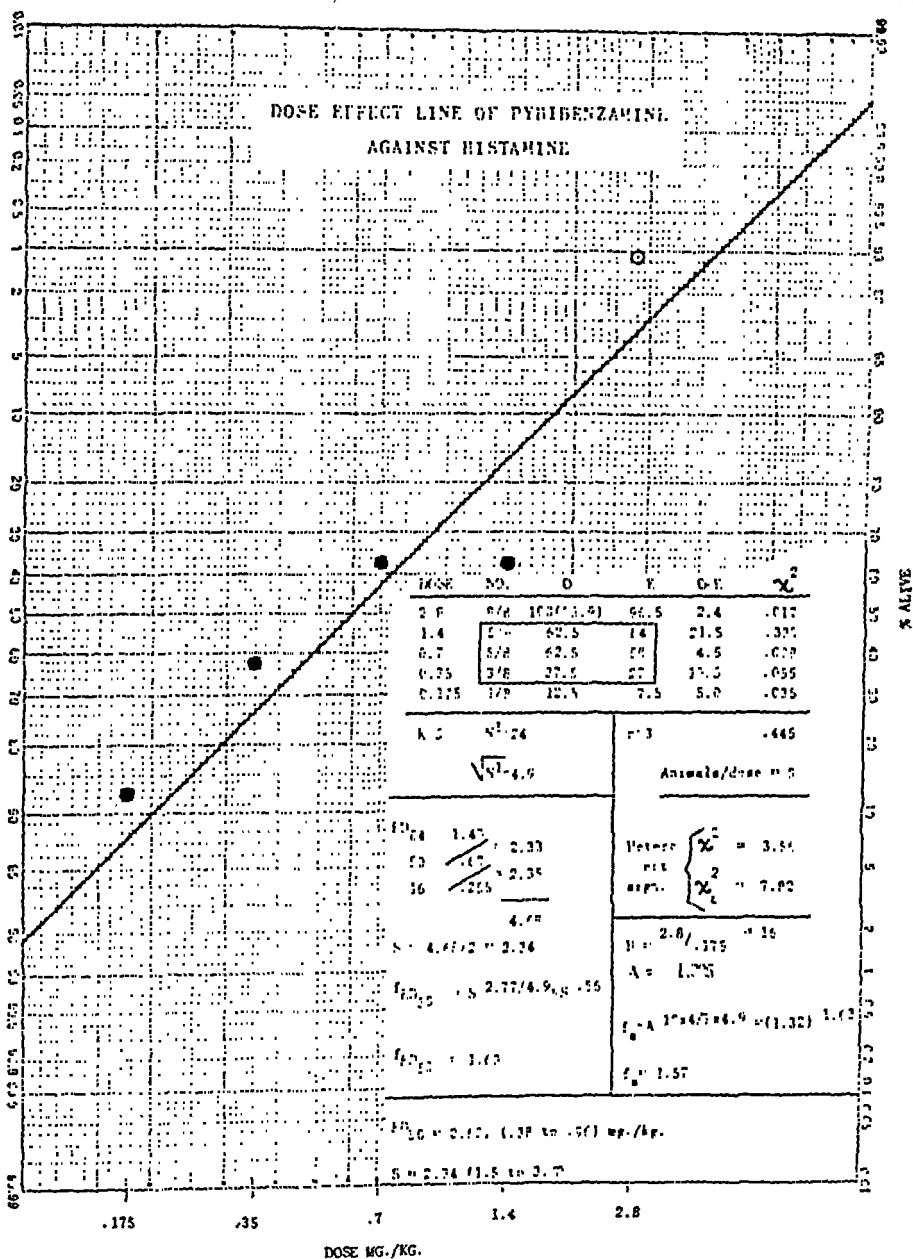


FIGURE 2

to be satisfactory for all ordinary purposes. The new method in some respects is undoubtedly more accurate since not only can a poorly fitted line be detected and improved but also significant heterogeneity, if present, will be found.

TABLE 1  
Corrected Values\* of 0 or 100 per cent Effect (Body of Table)  
Corresponding to Expected Values (Margins)

EXPECTED	0	1	2	3	4	5	6	7	8	9
0	—	0.3	0.7	1.0	1.3	1.6	2.0	2.3	2.6	2.9
10	3.2	3.5	3.8	4.1	4.4	4.7	4.9	5.2	5.5	5.7
20	6.0	6.2	6.5	6.7	7.0	7.2	7.4	7.6	7.8	8.1
30	8.3	8.4	8.6	8.8	9.0	9.2	9.3	9.4	9.6	9.8
40	9.9	10.0	10.1	10.2	10.3	10.3	10.4	10.4	10.4	10.5
50	—	89.5	89.6	89.6	89.6	89.7	89.7	89.8	89.9	90.0
60	90.1	90.2	90.4	90.5	90.7	90.8	91.0	91.2	91.4	91.6
70	91.7	91.9	92.2	92.4	92.6	92.8	93.0	93.3	93.5	93.8
80	94.0	94.3	94.5	94.8	95.1	95.3	95.6	95.9	96.2	96.5
90	96.8	97.1	97.4	97.7	98.0	98.4	98.7	99.0	99.3	99.7

\* These values are derived from the maximal and minimal corrected probits of Bliss (1).

TABLE 2  
Values\* of  $t$  and  $(Chi)^2$  for  $p = .05$

DEGREES OF FREEDOM	$t$	$(chi)^2$
1	12.7	3.84
2	4.3	5.99
3	3.18	7.82
4	2.78	9.49
5	2.57	11.1
6	2.45	12.6
7	2.36	14.1
8	2.31	15.5
9	2.26	16.9
10	2.23	18.3

\* Values of "students"  $t$  and  $(Chi)^2$  for  $p = .05$  are the same as may be found in more extensive tables such as those in (17).

TABLE 3\*

% EFFECT, Y	X
16 or 84	1.00
10 or 90	1.30
5 or 95	1.65
2 or 98	2.05
1 or 99	2.35

\* Other values of X may be obtained from any table relating deviations and areas of the normal curve, such as (17).

All approximate methods without exception have one or more weak points. The inadequacy of these methods becomes evident when they are applied to an

unbalanced or truncated set of data. Thus in the more exact method of Bliss (1), the confidence limits are corrected for the deviation (caused by unbalance), of the mean probit from 5.0. Due to the nature of the weighting coefficients this correction is of little significance unless the degree of truncation is rather large. Thus, in the case of any approximate method, the confidence limits will tend to be underestimated when the maximum observed effect is 70 per cent or less. This weakness common to all such methods is usually overlooked and the experimenter should avoid applying an approximate method to very poorly balanced experiments. In such cases repetition of the experiment or the use of the more exact procedure of Bliss (1) is indicated. It cannot be sufficiently emphasized, however, that a statistical method is in no way a substitute for a good experiment.

The name slope function has been applied to  $S$ , the antilogarithm of the quantity designated as  $s$  in Bliss' notation, or  $\lambda$  in Gaddums notation (18), where  $s$  or  $\lambda$  is the standard deviation of the logarithms of the individual effective doses. Our choice was based on the use of  $S$  for purposes customarily served by the slope constant  $b$ , and we are not aware of any existing designation for the antilogarithm of the standard deviation.

Since the dose-per cent effect curve is encountered so frequently in biological and occasionally even in non-biological fields, this rapid approximate method should be of help to the many individuals who have not the time, desire nor facilities for complex mathematical treatment of this kind of data.

#### SUMMARY

1. A rapid graphic method for approximating the Median Effective Dose and the Slope of dose-per cent effect curves is presented. Confidence limits of both of these parameters for 19/20 probability are given by the method. In addition, confidence limits for any other probability or for a dose other than the Median Effective Dose are readily estimated.

2. The data are used throughout the method in their original form without transformation to logarithms and probits.

3. An effective means for plotting and using 0 and 100 per cent effects is provided.

4. The calculations have been simplified by means of nomographs to the extent that a slide rule is a convenience but not a necessity.

5. A simple means is provided for detecting a poorly fitted line or significantly heterogeneous data. In the former case, the line may be refitted; in the latter, the confidence limits are corrected for the degree of heterogeneity.

6. The method provides means for the rapid test of parallelism of two curves and easy computation of relative potency with its confidence limits.

7. Although the method is rapid (10-15 minutes), its accuracy is commensurate with the nature of dose-per cent effect data.

## APPENDIX

**A Source or derivation of formulae used in the method** The revised method uses in modified form (1) The approximations developed by Litchfield and Fertig (13) for obtaining confidence limits of the parameters of a dose per cent effect curve, and (2) the method for  $(Chi)^2$  proposed by Wilcoxon and McCallan (14) The corrections used in the event of heterogeneity, the method for using 0 and 100 per cent effects, and the test for significant differences between values are derived from conventional procedures (1) The formula for obtaining approximate confidence limits of doses giving per cent effects other than 50 per cent is derived from that for the variance of the  $\log ED_{50}$  as given by Bliss (1)

The following table shows some of the relationships between formulae used in the revised method and their equivalents after transformation to the logarithm probit system

Arithmetic method	Log probit method
$ED_{50}$	$\log ED_{50}$
$fED_{50}$	$1.96SE_{\log ED_{50}}$
$S$	$\log S$ or $s$ or $1/b$
$f_s$	$1.96S E_s$ or $1.96s^2 S E_b$
$A/B$	$\log A - \log B$
$f_{A/B}$	$1.96\sqrt{(SE_{\log A})^2 + (SE_{\log B})^2}$
$fED_{Y\%}$	$1.96\sqrt{SE_{\log ED_{50}}^2 + [SE_s(y - 50)]^2}$

Corrected effect for 0 or 100 per cent

Maximal or minimal corrected probit

**B The parameters and confidence limits of a dose per cent effect line on logarithmic probability paper** 1 *The median effective dose  $ED_{50}$*  This is the dose indicated by the line to cause 50 per cent of the animals or items to react or not, to live or die, to be positive or negative, to fit into a category or not, etc Dose is used in the abstract sense and may be dose, time, size, distance, etc

2 *The slope function of the line  $S$*  This is the fold change in dose required to produce a unit standard deviation change in response along the line Thus

$$S = \text{antilog of } 1/b, s, \text{ or } \frac{X_1 - X_2}{Y_1 - Y_2}$$

where  $b$  and  $s$  are, respectively, the slope constant and standard deviation of a line relating  $\log$  dose  $X$ , and probit per cent effect  $Y$  Since  $s$  is actually the difference between two particular  $\log$  doses, its antilog, the slope function  $S$ , is the ratio of the arithmetic value of those doses

3 *The factor of  $fED_{50}$  for obtaining 19/20 confidence limits of the  $ED_{50}$*  This factor, using the notation of Litchfield and Fertig, is derived as follows

$$SE_{\log ED_{50}} = \frac{s}{\sqrt{N'/2}} \quad (1)$$

where  $s$  is the difference between two  $\log$  doses whose expected effects, as indicated by the line, differ by 1.0 probit and  $N'$  is the total number of animals or items tested between the  $\log$  dose limits corresponding to expected probits 4.0 and 6.0

Multiplying (1) by 1.96, simplifying and taking the antilog gives

$$fED_{50} = S^{2.77/\sqrt{N'}} \quad (2)$$

where  $S = \text{antilog } s$  and  $N'$  is now the total number of animals or items tested between arithmetic dose limits corresponding to expected 16 and 84 per cent effect

The slope function  $S$  can be obtained from the line on logarithmic probability paper by any of the following expressions but (3) is preferable

$$S = ED_{84}/ED_{16} \quad \text{or} \quad ED_{67}/ED_{33} \quad \text{or} \quad ED_{90}/ED_{10}$$

$$S = \frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2} \quad (3)$$

The factor for the  $ED_{50}$  can be reduced to the expression:

$$f_{ED_{50}} = S^{exp.}$$

where the exponent is  $2.77/\sqrt{N'}$ . The value of  $f_{ED_{50}}$  can then be read from the fractional power Nomograph No. 2.

4. The factor  $f_S$  for obtaining limits for 19/20 probability of the slope function  $S$ . This factor is derived from the approximation of Litchfield and Fertig to the standard error of the slope constant,  $b$ .

Since:

$$s = 1/b \text{ and } s^2 = 1/b^2$$

By differentiation:

$$ds = -b^{-2} db = -(1/b^2) db$$

Then by substitution:

$$ds = -s^2 db \text{ or}$$

$$S.E._s = s^2 S.E._b \quad (4)$$

The minus sign can be dropped because it merely signifies the reciprocal relation between  $s$  and  $b$ .

The approximation to the standard error of  $b$  for limits for 19/20 probability is given by:

$$1.96 S.E._b = \frac{7.85}{\sqrt{N'/2} \text{LK}/K - 1}$$

where  $L$  and  $K$  refer, respectively, to the logarithmic dosage range of the experiment and the number of doses tested.  $N'$  is the same as defined above. This can be simplified to give:

$$1.96 S.E._b = \frac{11.1(K - 1)}{LK\sqrt{N'}} \quad (5)$$

By substitution of (5) in equation (4): and rewriting:

$$1.96 S.E._s = \frac{11.1 s^2 (K - 1)}{LK\sqrt{N'}} = \frac{1.1 s^2}{L} \times \frac{10(K - 1)}{K\sqrt{N'}}$$

$$\text{Let: } A = \text{antilog } \frac{1.1 s^2}{L},$$

$$R = \text{antilog } L$$

$$\text{and, since } S = \text{antilog } s,$$

$$\text{then } A = \text{antilog } \frac{1.1(\log S)^2}{\log R} \quad (6)$$

$$\text{and } f_S = \text{antilog } 1.96 S.E._s = A^{10(K-1)/K\sqrt{N'}} = A^{exp.} \quad (7)$$

The value of  $S$  is known from equation (3) and the value of  $R$  is given by the ratio:  $R = \text{largest/smallest dose plotted}$ . In order to eliminate the use of logarithms, Nomograph No. 3, having scale values in units of  $S$  and  $R$ , was constructed to solve equation (6) to give the value of  $A$ .  $K$  is the number of doses plotted and  $N'$  has already been defined. The factor for  $S$  can then be read from Nomograph No. 2 using the value of  $A$  and its exponent.

The confidence limits for the parameters  $ED_{50}$  and  $S$  are obtained by using the factors  $f_{ED_{50}}$  and  $f_S$  as follows:

$$\left. \begin{array}{l} \text{Parameter} \times f = \text{upper} \\ \text{Parameter}/f = \text{lower} \end{array} \right\} \text{limit for 19/20 probability.}$$

C *Additions to the basic method* 1 *Use of 0 and 100 per cent effects* Fisher (cited by Bliss, 1) has shown that the most likely value for 0 or 100 per cent effects is the minimal or maximal corrected probit, the exact value of which is determined by the expected probit obtained from the line on the log dose probit graph. The equivalent procedure for 0 or 100 per cent effect in the case of plotting on logarithmic probability paper is made possible by means of a table relating the expected per cent effect, indicated by the line, to the minimal or maximal corrected per cent effect. These corrected values have been interpolated and converted to percentages from the original table of corrected probits (1).

The procedure for using 0 or 100 per cent effects consists of (a) plotting the data on logarithmic probability paper, omitting 0 or 100 per cent effects, and fitting a temporary line with transparent straight edge or triangle, (b) reading the expected per cent effect indicated by the straight edge at doses where 0 or 100 per cent effect was observed, (c) converting the expected per cent effect to a corrected value by means of table 1 and plotting this corrected value, (d) drawing a line through the completely plotted data.

2 *Recognition of heterogeneous data* *Test of the line for "Goodness of Fit"* The nomographic calculation of  $(\text{Chi})^2$ , previously reported by Wilcoxon and McCallan (14), was modified slightly and incorporated into the revised method. In this portion of the procedure, the expected per cent effects from the line are listed opposite the observed per cent effect and a list of differences between observed and expected per cent effects made. For each set of a difference and the corresponding expected per cent effect, a  $(\text{Chi})^2$  value based on one animal or item is read from Nomograph No. 1. The total of these  $(\text{Chi})^2$  values multiplied by the average number of animals or items per dose is the  $(\text{Chi})^2$  of the dose effect line. The degrees of freedom,  $n$ , are two less than the number of points plotted, i.e.,  $n = K - 2$ . By comparison of this to the value of  $(\text{Chi})^2$  for probability of .05 and  $n$  degrees of freedom, significant heterogeneity can be recognized. In the event of significant heterogeneity a better fitting line can often be drawn, and if not, the equations for the factors of the parameters are modified to include this additional variation. For this modification the value of "students"  $t$  for a probability of .05 and  $n$  degrees of freedom must be used. For convenience in using the method, the important values of "students"  $t$ , and  $(\text{Chi})^2$  for  $p = .05$  and various degrees of freedom are given in table 2. Other values may be found in more extensive tables such as are given by Snedecor (15).

When significant heterogeneity is found the factors of the parameters are obtained by the following equations, whose nomographic solution is the same, however, as described above.

For heterogeneous data

$$f_{ED_{50}} = S^{1.4t\sqrt{(\text{Chi})^2/nN}} = S^{\text{exp}} \quad (8)$$

$$f_B = A^{[5.1t(K-1)\sqrt{(\text{Chi})^2/nN}]/K} = A^{\text{exp}} \quad (9)$$

All symbols have the same significance as noted above.

The change which has been made in the exponents of both of the factors in order to correct for heterogeneity is the conventional multiplier (16) while the value of "t" replaces the 1.96 which was previously introduced into the exponent and must now be divided out again. Thus, for limits for 19/20 probability,

$$(\text{Exp homogeneous}) \times \frac{t\sqrt{(\text{Chi})^2/n}}{1.96} = \text{Exp heterogeneous}$$

Nomograph No. 1 computes  $(\text{Chi})^2$  for a single item as

$$(\text{Chi})^2 = \frac{(\text{Observed} - \text{expected per cent effect})^2}{(\text{Expected effect})(100 - \text{expected effect})} \quad (10)$$

which is derived from the expression used by Wilcoxon and McCallan (14) for nomographic solution of  $(\text{Chi})^2$  for 100 items



3. *Comparison of two dose-effect curves: The test for parallelism and the ratio of potencies.* In the method of Bliss (1) and the approximate method of Litchfield and Fertig (13), the significance of differences between the parameters of two curves was tested by\* computing the standard error of the difference as:

$$S.E._{Diff.} = \sqrt{(S.E._1)^2 + (S.E._2)^2}$$

In the revised method, the equivalent arithmetic procedure is used; that is, in place of a difference between two logarithmic quantities, the ratio of the quantities themselves is used. Furthermore, in place of the standard error of a log quantity, the factor of the quantity itself is used since, as already noted, the factor is the antilog of the Standard Error.

Thus, for limits for 19/20 probability, in place of  $1.96 S.E._{Diff.} = \sqrt{(\log f_1)^2 + (\log f_2)^2}$ , the following equation is used:

$$f_{ratio} = \text{antilog } \sqrt{(\log f_1)^2 + (\log f_2)^2} \quad (11)$$

To eliminate logarithms, Nomograph No. 4 was constructed having scale values in units of  $f_1$  and  $f_2$  which, if connected by a straight edge, permit reading  $f_{ratio}$  on the intersected center scale. The procedure is the same for both the factor of the ratio of the slope functions  $S_1/S_2$  or the ratio of potencies  $ED_{50_1}/ED_{50_2}$ .

The factor of the ratio may be used as already described to obtain the limits of the ratio for 19/20 probability. If this is done, it is evident that if the lower limit is greater than 1.0 the ratio is significant. However, the lower limit can exceed 1.0 only if the value of the ratio exceeds that of the factor.

Therefore, two curves may be considered parallel if  $S.R.$ , the slope function ratio, *does not exceed* its factor,  $f_{S.R.}$ ; and two potencies may be considered significantly different if  $P.R.$ , the potency ratio, *exceeds* its factor  $f_{P.R.}$ .

4. *Confidence limits for 19/20 probability of doses other than the median effective dose,  $ED_{50}$ .* A satisfactory approximation to the limits of errors of  $ED_Y$  (where  $Y$  is a response other than 50 per cent) can be derived from the expression for the (standard error)<sup>2</sup> of a log dose whose probit response  $y$  deviates from the mean probit,  $\bar{y}$  of the experiment. This expression which Bliss (1) gives can be written as:

$$S.E._{ED_Y}^2 = (S.E._{bs}^2)(y - \bar{y})^2 + V_{bs}^2 \quad (12)$$

It has been shown that:

$$S.E._{bs}^2 = S.E._s \quad (4)$$

Furthermore, the first of the approximations of the Litchfield and Fertig method was derived as:

$$V_{bs}^2 \sim S.E._{log ED_{50}}^2 \sim \frac{2s^2}{N'} \quad (1A)$$

By substitution of (4), (1A), and the approximation:

$$(y - \bar{y}) \sim (y - 5.0) = X$$

equation (12) becomes:

$$SE_{log ED_Y}^2 = [S.E._s(X)]^2 + S.E._{log ED_{50}}^2$$

which is the same as:

$$(\log fed_Y)^2 = (\log [(f_s)^X])^2 + (\log fed_{50})^2$$

the antilogarithm of the root of this expression is in a form suitable for use in the revised method dealing with arithmetic doses and per cent responses, namely:

$$fed_Y = \text{antilog } \sqrt{(\log [f_s^X])^2 + (\log fed_{50})^2} \quad (13)$$

since Nomograph No. 4 solves expressions of this type. The value of  $X$  can be read from any table relating deviations and areas of the normal curve (17). Thus, 16 and 84 per cent effect both depart from 50 by 34. The area, 34 per cent, corresponds to a deviation  $X$ , of 1.00 in the table cited above. For convenience certain commonly used values of  $X$  are given in table 3.

In certain cases one may desire to adopt confidence limits for probabilities other than 19/20. This may be done easily by using the proper multiplier for the exponents of  $S$  and  $A$  before reading  $fed_{10}$  and  $f_9$  from Nomograph No. 2. For probabilities commonly used these multipliers are listed below:

<i>For p of:</i>	<i>Multiply exponent by:</i>
0.32 (2/3 odds)	0.51
0.10 (9/10 odds)	0.84
0.05 (19/20 odds)	1.00
0.02 (49/50 odds)	1.19
0.01 (99/100 odds)	1.31
0.001 (999/1000 odds)	1.68

### REFERENCES

- (1) BLISS, C. I.: *Quart. J. Pharm. and Pharmacol.*, **11**: 192, 1938.
- (2) BERKSON, J.: *J. Am. Stat. Assoc.*, **39**: 357, 1944; **41**: 40, 1946.
- (3) KNUDSEN, L. F., AND CURTIS, J. M.: *J. Am. Stat. Assoc.*, **42**: 282, 1947.
- (4) DRAGSTEDT, C. A., AND LANG, V. F.: *THIS JOURNAL*, **32**: 215, 1928.
- (5) BEHRENS, B.: *Arch. exper. Path. u. Pharmacol.*, **140**: 237, 1929.
- (6) REED, L. J.: *Biological Effects of Radiation*, 227-51, 1936, McGraw-Hill Book Co., Inc.
- (7) REED, L. J., AND MUENCH, H.: *Am. J. Hygiene*, **27**: 493, 1938.
- (8) KARBER, G.: *Arch. exper. Path. u. Pharmacol.*, **162**: 480, 1931.
- (9) IRWIN, J. O., AND CHEESEMAN, E. A.: *J. Hygiene, Comb.* **39**: 574, 1939.
- (10) THOMPSON, W. R.: *Bact. Reviews*, **11**: 115, 1947.
- (11) MILLER, L. C., AND TAINTER, M. L.: *Proc. Soc. exper. Biol. and Med.*, **57**: 261, 1944.
- (12) DEBEER, E. J.: *THIS JOURNAL*, **85**: 1, 1945.
- (13) LITCHFIELD, J. T., JR., AND FERTIG, J. W.: *Bull. Johns Hopkins Hosp.*, **69**: 276, 1941.
- (14) WILCOXON, F., AND MCCALLAN, S. E. A.: *Contrib. of the Boyce Thompson Inst.*, **10**: 329, 1939.
- (15) SNEDECOR, G. W.: *Statistical Methods*, Iowa State College Press, Ames, Iowa, pages 58 and 163, 1940.
- (16) FINNEY, D. J.: *Probit Analysis*, Cambridge University Press, page 60, 1947.
- (17) *Mathematical Tables from Handbook of Chemistry and Physics*, Chemical Rubber Publishing Company, Cleveland, Ohio, 7th Ed., p. 200, 1941. In this table  $X$  is listed as  $t$ .
- (18) GADDUM, J. H.: *Med. Res. Council Spec. Report*, **183**, 1933.

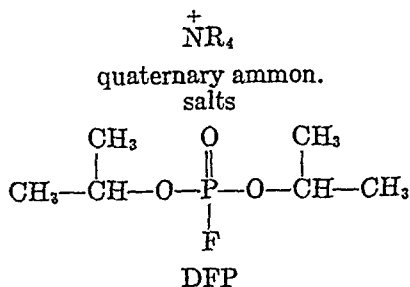
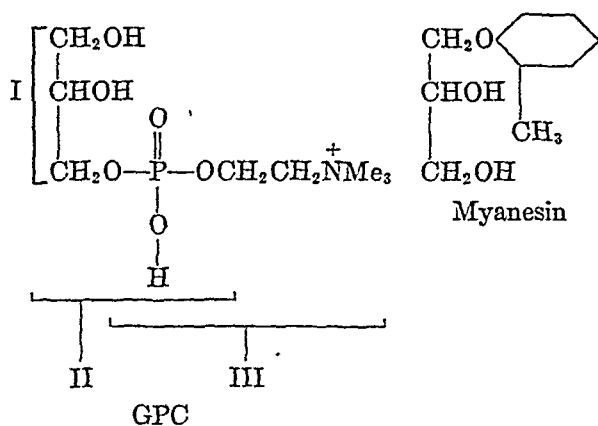
# THE INHIBITION OF ACID PHOSPHATASE OF SHEEP BRAIN<sup>1</sup>

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The large concentration of acid phosphatases in nerve tissue led the authors to investigate the possibility that drugs known to act through nervous mechanisms may owe a part of their action to a direct effect on acid phosphatases. Three types of drugs of particular interest were quaternary ammonium salts, phosphoric or pyrophosphoric esters, such as tetraethyl pyrophosphate and diisopropylfluorophosphate (DFP), and glycerol ethers, such as Myanesin. As may be seen from the accompanying diagram, these compounds have structures similar to the glycerophosphorylcholine (GPC) or lecithin molecule. It is



readily noted that a structural similarity exists between Myanesin and Part I, between DFP and Part II, and between quaternary ammonium salts and Part

<sup>1</sup> A portion of these data were abstracted from a thesis presented by Sally D. Holtan to the Graduate School of Western Reserve University in partial fulfillment of the requirements for the degree of Master of Science.

<sup>2</sup> Fellow of the Scandinavian-American Foundation, 1947-48.

III of the GPC molecule. It seemed conceivable, therefore, that these types of compounds might act through a competitive mechanism to affect the action of enzymes that hydrolyze corresponding portions of the GPC molecule.

An investigation was planned to test this hypothesis by studying the effects of known inhibitors of acid glycerophosphatase and cholinephosphatase, testing qualitatively a group of drugs believed to act by modifying nerve impulse transmission, and finally, by examining in more detail the effect of any new inhibitors of the enzyme system.

A survey of the literature revealed no kinetic data on the effects of known inhibitors of acid phosphatases from which could be deduced the nature of inhibition, although a number of substances have been reported to inhibit acid phosphatases from various sources. In most cases the hydrolysis time had been greater than is now generally accepted for inhibition studies. Most enzyme preparations were crude extracts, and no conclusive evidence has been presented to prove whether the acid phosphatases from the various sources, and those hydrolyzing glycerophosphate and cholinephosphate, are identical.

In this study a phosphatase preparation from nerve tissue was desired, and the extract of Giri and Datta (1) was selected as most suitable. Preliminary tests showed that acid phosphatase extracts prepared in this manner were surprisingly stable, there being no change in activity after one month's storage at refrigerator temperatures. Also, subsequent solutions prepared in the same manner from a given lot of acetone-dried brain powder gave almost identical activities. These extracts were found to hydrolyze both glycerophosphate and phosphorylcholine, and exhibited the same pH optima (5.0 and 9.5) as reported by Giri and Datta.

**EXPERIMENTAL.** *Qualitative tests for inhibition.* A number of chemicals were tested qualitatively for inhibitory effects on the enzyme system, using both phosphorylcholine (synthesized by the method of Baer, 2) and glycerophosphate (Eastman) substrates. Relatively high concentrations of test substances were used, and inhibition was considered to be positive when test substances caused a decrease of 20 per cent or more in liberated phosphate when compared with controls.

Digests were prepared as follows:  $1.2 \times 10^{-3}$  Moles of substrate (sodium glycerophosphate or sodium phosphorylcholine) were dissolved in about 90 cc. water, and the solution was adjusted to pH 5.0 by addition of acetic acid. One hundred cc. of 0.2 M acetate buffer and sufficient water to make 200 cc. were added. For each digest 5 cc. of the adjusted substrate, 1 cc. of 4.29 M test substance, and 1 cc. of enzyme were mixed. These mixtures contained as final concentrations: substrate— $4.29 \times 10^{-3}$  M, test substance— $4.29 \times 10^{-3}$  M, acetate buffer—0.1 M. The tubes containing the digests were stoppered and placed in the incubator at 37°C. for three hours. At the end of this time, 1 cc. of 20 per cent trichloroacetic acid was added to each digest, the mixture was centrifuged, and 5 cc. of the centrifugate was analyzed for free phosphate by the method of Lowry and Lopez (3). Tests were performed in triplicate with a triplicate series of blanks (1 cc. water replacing enzyme) and of controls (1 cc. water replacing test substance).

The following compounds were tested in this manner: sodium sulfate, sodium pyrophosphate, sodium cyanide, sodium fluoride, idoacetic acid, alanine, glutamic acid, p-aminobenzoic acid, creatine, cysteine, cystine, glutathione, ascorbic acid, acetylcholine, carbachol, tetramethyl ammonium bromide, tetraethyl ammonium bromide, choline, urethane, tetraethyl pyrophosphate (Nifos-T), triethyl phos-

phate, tri-*o*-cresylphosphate, myanesin, procaine, sulfanilamide, pilocarpine, and DFP. Four of these compounds, sodium sulfate, sodium pyrophosphate, sodium fluoride, and DFP, inhibited hydrolysis of both substrates, while sodium fluoride caused loss of enzyme activity which varied with time of contact between enzyme and cyanide, probably protein denaturation. All other compounds tested were without effect. The choice of test substances was limited to those which did not interfere with the color measurement, making necessary the exclusion of a number of alkaloids and arsenic compounds that may have been of interest.

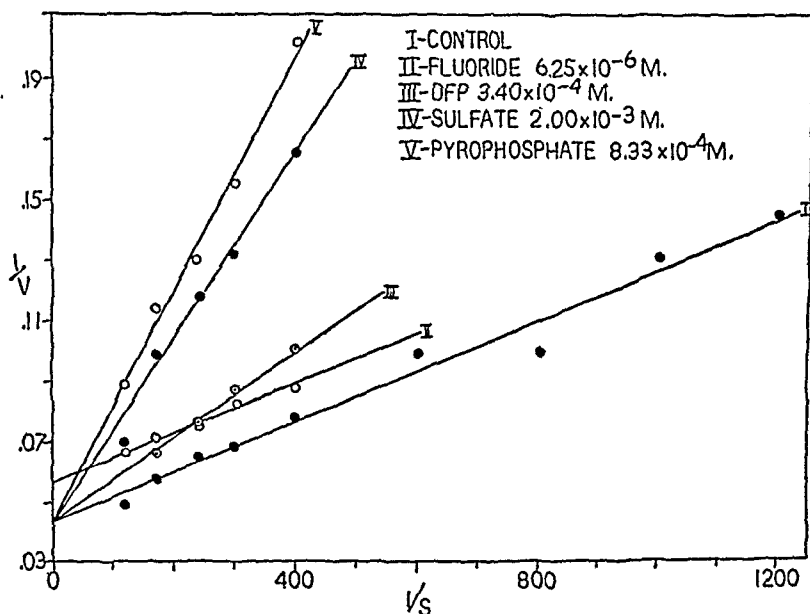


Fig. 1. Lineweaver-Burke plot of acid glycerophosphatase activity with and without inhibitors. Initial velocity ( $V$ ) expressed as micrograms P liberated/30 minutes, and substrate concentration ( $S$ ) as moles/liter.

*Kinetic studies of inhibition.* Inasmuch as the response of the extracts to inhibitors was qualitatively the same whether glycerophosphate or phosphorylcholine were the substrate, kinetic studies were limited to the hydrolysis of beta-glycerophosphate.

Initial curves obtained by measuring the hydrolysis of the two substrates at time intervals of 30 minutes for periods up to six to eight hours appeared to be linear up to three hours, agreeing with the curves of Giri and Datta, but later experiments conducted in this laboratory with 30 minutes hydrolysis time gave rates vastly different, and it is apparent that the original curves were misleading due to failure to expand them at lower time values.

Digests were prepared by adding 2 cc. of a solution of sodium beta-glycerophosphate of proper concentration, 1 cc. of test solution, and 1 cc. of enzyme solution to 2 cc. of 0.2 *M* acetate buffer pH 5.0, all solutions being brought to 37°C. before mixing. Digests were incubated for a period of 30 minutes the values obtained being taken as initial velocities.

Substrate concentrations were varied between  $8.33 \times 10^{-4}$  and  $8.33 \times 10^{-3}$  for the control curve. A plot of hydrolysis vs. substrate concentration showed a decline in free phosphate at and above substrate concentrations of  $1 \times 10^{-2}$  M. No attempt was made to determine whether the drop was due to substrate inhibition or interference with measurement. The minimum concentration that gave accurately measurable phosphate content after inhibition was  $1.5 \times 10^{-3}$  M. The Lineweaver-Burke method (4) was used to evaluate enzyme-substrate dissociation constants ( $K_s$ ) and enzyme-inhibitor dissociation constants ( $K_i$ ),

TABLE 1  
*Effect of inhibitors on acid glycerophosphatase activity kinetic studies*

SUBSTRATE CONCENTRATION (S) $\times 10^{-3}$	INITIAL VELOCITY—MICROGRAMS P/30 MINUTES				
	Control	Fluoride $6.35 \times 10^{-4}M$	Sulfate $2.00 \times 10^{-4}M$	Pyrophosphate $8.33 \times 10^{-4}M$	DFP $3.4 \times 10^{-4}M$
0.83	6.94				
1.00	7.69				
1.25	10.07				
1.67	10.19	9.90	5.31	—	7.15
2.50	12.97	11.50	6.09	4.89	10.01
3.33	14.80	12.25	7.64	6.54	11.60
4.17	15.61	13.15	8.55	7.77	13.46
5.83	17.46	14.12	10.20	8.88	15.81
8.33	20.29	15.29	—	11.46	—
10.0*	13.73				
25.0*	5.81				
Mean Dev. from Calc.	0.40	0.22	0.32	0.25	0.39
$V_m$	23.9	18.2	23.9	23.9	23.9
$K_s$	0.00203	0.00155	—	—	—
$K_i$	—	—	0.000752	0.000235	0.000504
Type of Inhibition	—	Non-com- petitive	Competitive	Competitive	Competitive

\* These values not included in deviations, nor on graph.

and for the determination of competitive or noncompetitive mechanisms of action. Results reported in table 1 are average values for four or more determinations which agreed within 10 per cent of one another. The resultant curves are shown in fig. 1. In order to determine the accuracy of the curves, initial velocities were calculated, using the constants obtained from the graph, and the

$$\text{formula } v = \frac{V_m S}{K_s + S} \text{ for uninhibited and } v = \frac{V_m S}{K_s + S + \frac{K_s I}{K_i}} \text{ (5) for inhibited}$$

enzyme activity. The mean deviation of experimental from calculated values are given in table 1.

Special precautions had to be taken with DFP solutions to avoid hydrolysis. This was done by preparing a stock solution of DFP in anhydrous isopropanol and diluting it with distilled water immediately before use. Preliminary tests showed that the amounts of isopropanol present in the digests did not affect enzyme activity.

CONCLUSIONS. The results of this work show that, in spite of the structural similarity between a number of the drugs tested and the metabolite, glycerophosphorylcholine, these drugs, with the exception of DFP, do not directly affect the acid glycerophosphatase or cholinephosphatase action of brain extracts. In addition, the facts that the concentrations of DFP required for effective inhibition were considerably higher than those necessary for inhibition of cholinesterase (6), and that drugs having physiological action similar to DFP, such as tetraethyl pyrophosphate, tri-*o*-cresyl phosphate, and eserine, had no effect on the acid phosphatase activity, make it appear unlikely that DFP owes its primary physiological action to inhibition of this enzyme system.

#### SUMMARY

1. Structural similarity between certain drugs known to act on the nervous system and glycerophosphorylcholine suggested possible competitive action, and a number of these drugs were tested for their effect on the hydrolysis of beta-glycerophosphate and phosphorylcholine by brain extracts at pH 5.0. Only one of the drugs tested (DFP) inhibited the enzyme system, and it was concluded that acid phosphatases are not the site of action of these substances.

2. Fluoride inhibited noncompetitively; sulfate, pyrophosphate, and DFP inhibited acid glycerophosphatase competitively, their  $K_i$  values being  $7.52 \times 10^{-4}$ ,  $2.35 \times 10^{-4}$ , and  $5.04 \times 10^{-4}$ , respectively.

#### REFERENCES

1. GIRI, K. V., AND DATTA, N. C.: *Biochem. J.*, **30**: 1089, 1936.
2. BAER, E.: *J. Am. Chem. Soc.*, **69**: 1253, 1947.
3. LOWRY, O. H., AND LOPEZ, J. A.: *J. Biol. Chem.*, **162**: 421, 1946.
4. LINEWEAVER, H., AND BURKE, D.: *J. Am. Chem. Soc.*, **56**: 658, 1934.
5. HALDANE, J. B. S.: *Enzymes*, London, 1930, p. 39, 46.
6. BRAUER, R. W.: *THIS JOURNAL*, **92**: 162, 1948.

# ANTIBIOTIC LACTONES AND SYNTHETIC ANALOGS. II. CARDIOTONIC EFFECTS ON THE ISOLATED FROG HEART<sup>1,2</sup>

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In a preliminary study of the cardiotoxic effects of several lactone derivatives (1), some of which have been shown to possess distinct antibacterial and anti-protozoan activity, it was observed that many of the agents evoked an initial positive inotropic response in the isolated frog heart, which persisted for long periods before the ultimate systolic standstill. This observation suggested an investigation of the cardiotonic potency of these agents.

The approach to the problem of synthetic cardiotonic substances through the lactone structure has been utilized by several investigators. All of the active constituents of the cardiac glycosides (digitalis, strophanthin, and squill) have been shown to contain an unsaturated gamma- or delta-lactone substituent on C-17 of the cyclopentanoperhydrophenanthrene nucleus. It has been repeatedly demonstrated that this lactone moiety with its double bond is essential for the cardiac activity, although it is recognized that a favorable configuration of the steroid nucleus can enhance the action (2). Two independent lines of research have been pursued recently by Chen and co-workers, and Kraye, Mendez and associates with a view toward discovering new cardiotonic substances among synthetic lactones. Preliminary studies by the Chen group failed to reveal any digitalis-like action in four simple lactones by the frog lymph-sac toxicity method (3). Later, however, the same investigators successfully produced systolic standstill in the intact frog heart, similar to the digitalis effect, with several of a series of 27 lactones (4).

Kraye, on the other hand, approached the problem more directly from the cardiotonic point of view. Utilizing a modification of the Straub technique on the isolated frog heart, he was able to demonstrate an improvement in the contractions of the heart made hypodynamic with a low calcium perfusion fluid, by some of a series of lactones (5, 6). In several papers which followed, Kraye and his group continued to corroborate this lactone cardiotonic effect and attempted to explain its mechanism (7, 8).

It is the purpose of this work to determine the extent and possible mechanism

<sup>1</sup> The material presented here represents a portion of the data contained in a dissertation submitted on May 1, 1948 to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

<sup>2</sup> Aided in part by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

<sup>3</sup> National Institute of Health Research Fellow, 1947-1948. Present address, E. I. du Pont de Nemours & Co., Grasselli Chemicals Department, Wilmington, Delaware.



of the digitalis-like cardiotonic action of certain lactone derivatives on the isolated frog heart made hypodynamic by low calcium Ringer's solution. An attempt will be made to relate chemical structure with cardiotonic activity.

The compounds tested here were accumulated mainly from several industrial and academic laboratories. The sources are given in table I, where all the agents appear.

One compound, 3-methyl-5-carboxy-2-pentene-1,4-olide, was synthesized in this laboratory according to the method of Rinkes (9), with the aid and at the suggestion of Professor W. Bergmann of the Department of Chemistry at Yale University.

**METHODS.** The materials and methods used were essentially the same as those reported in an earlier work (1).

The normal perfusion fluid was the same as that used by Krayer and had the following composition: NaCl, 0.65 per cent; KCl, 0.014 per cent;  $\text{CaCl}_2$ , 0.011 per cent;  $\text{NaHCO}_3$ , 0.02 per cent. The low calcium solution contained all of the above salts in the same concentrations except the  $\text{CaCl}_2$ , which was decreased to 0.0055 per cent or one-half the normal amount.

All of the compounds were dissolved in 95 per cent ethyl alcohol to make up stock solutions varying from one to five per cent. Appropriate dilutions were made with the perfusion fluid prior to the experiments, and at no time did the alcohol concentration in the perfusion solution exceed one per cent.

The pH of all solutions was adjusted to that of the perfusion solution, which was maintained between pH 7.5 and 7.9. The experiments were carried out at room temperature which varied from 23°C. to 25°C.

Each experiment was executed in this manner. After excision of the heart, cannulation, and attachment with the recording lever, the preparation was perfused with normal Ringer's solution until a constant amplitude and rate of contraction was established. Then the heart was tested for sensitivity to the calcium ion depletion, by replacing the normal Ringer's with the low calcium solution until a state of hypodynamia existed, and then returning the heart to its original state by again perfusing with normal Ringer's. Following this, the hypodynamic state was again induced as above and, after a constant rate and amplitude of contraction had been established (usually within 5 minutes), the chemical agent was introduced into the low calcium solution and allowed in this way to perfuse through the hypodynamic heart.

**RESULTS.** The information sought in this investigation has been of a general nature, arising from a screening-type technique. In order to compare activities, a ratio has been established between the liminal millimolar positive inotropic concentration of ouabain and that of the other compounds. In this case the liminal concentration has been defined as that minimum concentration which restored the ventricular amplitude of 3 out of 4 hypodynamic hearts to 75 per cent or more of their normal amplitudes within 20 minutes.

The sources of error in this type of quantitative evaluation are well recognized—actual position on the dosage-response curve is not ascertained, and the threshold dose is dependent upon chance fluctuations in sampling, size of sampling, and the method of multiple dilution used to arrive at the minimum dosage sought. It was felt, however, that since the general order of potency of most of these compounds was feeble relative to that of ouabain, a more costly and time-consuming method was not warranted.

Relative cardiotonic activity of antibiotic lactones and synthetic analogs, and certain cardiac glycosides on the hypodynamic, isolated frog heart

COMPOUND (SOURCE)	CHEMICAL STRUCTURE	LIMINAL POSITIVE INOTROPIC CONCENTRATION		POTENCY RA- TIO (OUABAIN = 1)
		milligrams per 100 cc	millimoles	
Ouabain* Digitoxin Uzarin 2H <sub>2</sub> O		0.0125 0.0400 0.1000	0.000214 0.000524 0.001401	1.00 2.5 6.6
Butyrolac- tone (Cliffs Dow)		>100.00	—	—
Gamma val- erolactone (Mon santo)		>100.00	—	—
Delta capro- lactone (Am Cy- anamid, Calco Div.)		>100.00	—	—
Pantolac- tone		>100.00	—	—
2 keto 3 di- methyla- minometh- yl butyro- lactone (Sterling- Winthrop)		5.00	0.318	1489
2 keto 3 (3,3' di- methyl acryl) bu- tyrolac- tone (Vick and Ster- ling-Win- throp)		10.00	0.549	2565

TABLE 1—Continued

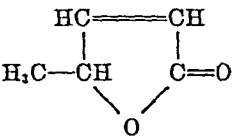
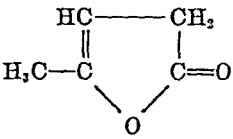
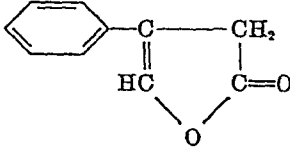
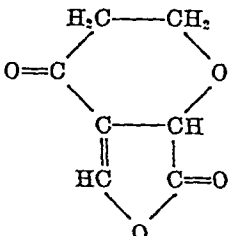
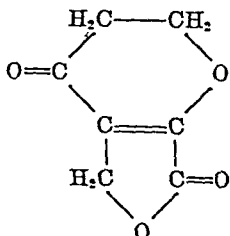
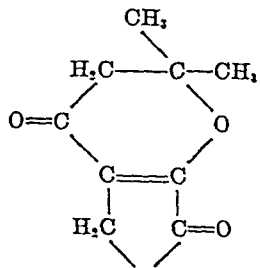
COMPOUND (SOURCE)	CHEMICAL STRUCTURE	LIMINAL POSITIVE INOTROPIC CONCENTRATION		POTENCY RA- TIO (OUABAIN = 1)
		milligrams per 100 cc.	millimoles	
2-pentene- 1,4-olide (Winthrop and Am. Cyanamid, Calco Div.)		10.0	1.020	4770
3-pentene- 1,4-olide (Winthrop and Am. Cyanamid, Calco Div.)		1.25	0.128	600
3-phenyl-3- butene- 1,4-olide (Sterling- Winthrop)		1.00	0.063	292
Clavacin (Dr. H. Raistrick, Lond. Sch. Hyg. Trop. Med., and Vick)		5.00	0.325	1518
Isoclavacin (Vick)		5.00	0.325	1518
Dimethyl- isoclavacin (Vick)		20.00	1.044	4879

TABLE 1—Continued

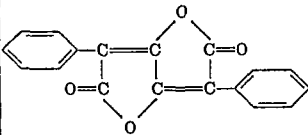
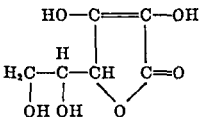
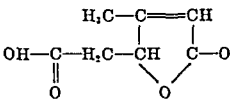
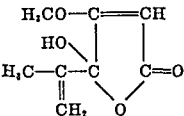
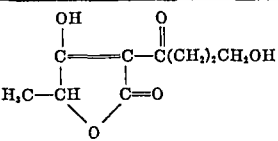
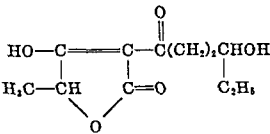
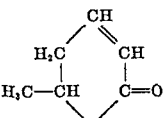
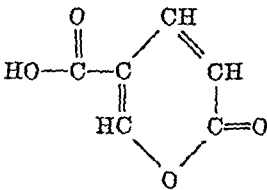
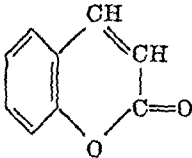
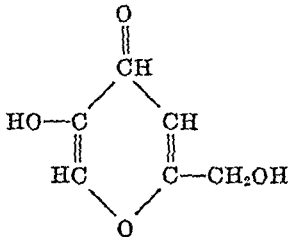
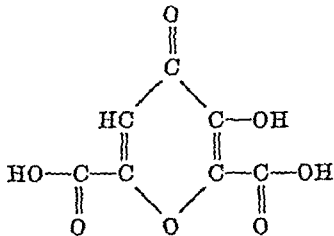
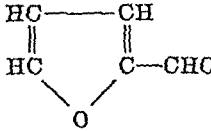
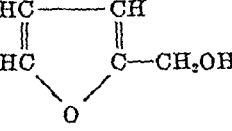
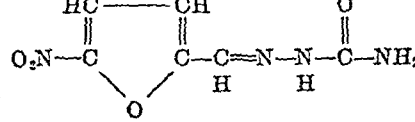
COMPOUND (SOURCE)	CHEMICAL STRUCTURE	LIMINAL POSITIVE INOTROPIC CONCENTRATION		POTENCY RA- TIO (OUABAIN = 1)
		milligrams per 100 cc.	millimoles	
Dilactone of pulvinic acid (Dr. W. Berg- mann, Yale Dept. of Chem.)		0.10	0.004566	21.3
l-Ascorbic acid		1.25	0.071	334
3-methyl-5- carboxy-2- pentene- 1,4-olide (Giarman)		20.00	1.282	6000
Penicillic acid (Dr. H. Rais- trick, Lond. Sch. Hyg. and Trop. Med.)		5.00	0.294	1374
Carolic acid (Dr. H. Raistrick, Lond. Sch. Hyg. and Trop. Med.)		10.00	0.500	2336
Terrestric acid (Dr. H. Rais- trick, Lond. Sch. Hyg. and Trop. Med.)		20.00	0.887	4145
Parasorbic acid (Am. Cyanamid, Calco Div.)		10.00	0.900	4206

TABLE 1—Continued

COMPOUND (SOURCE)	CHEMICAL STRUCTURE	LIMINAL POSITIVE INOTROPIC CONCENTRATION		POTENCY RA- TIO (OUABAIN = 1)
		milligrams per 100 cc.	millimoles	
Coumalinic acid (Ster- ling-Win- throp)		5.0	0.446	2000
Coumarin (Dr. W. Bergmann, Yale Dept. of Chem.)		20.00	1.370	6402
Kojic acid (Northern Reg. Res. Lab.)		10.00	0.714	3336
Meconic acid (Dr. W. Bergmann, Yale Dept. of Chem.)		1.25	0.068	309
Furfural (Quaker Oats)		>100.00	—	—
Furfuryl al- cohol (Quaker Oats)		>100.00	—	—
Nitrofura- zone (Eaton Labs.)		>0.80	—	—

\* Dehydrated ouabain: molecular weight = 584.

A summary of the results appears in table 1. The potency ratio listed is the denominator of the ratio of the activity of ouabain (as 1) to that of the agent under consideration. Thus, as this value increases, the potency decreases.

1. *Lactone series*—Of the saturated lactones studied two alone displayed any activity, and this was relatively slight. These compounds were 2-keto 3(3,3-dimethylacetyl)butyrolactone and 2-keto 3-dimethylaminomethylbutyrolactone, the latter being roughly twice as potent as the former. Despite their low order of activity, however, these agents were capable of restoring the hypodynamic heart to 100 per cent of its normal amplitude in concentrations of 5 to 10 mgm per 100 cc. The other saturated lactones were devoid of activity at the highest concentration tested, 100 mgm per 100 cc.

All of the unsaturated lactones exerted some positive inotropic effect. The most potent of these by far was the dilactone of pulvic acid, which caused 100 per cent recovery in concentrations ranging from 75 to 150 microgm per 100 cc. This high cardiotonic potency and the high relative cardiotoxicity of this drug, reported in an earlier paper (1), prompted a further study toward a precise measurement of its relative cardiotonic potency. Dosage response data from

TABLE 2

*Relative cardiotonic activities of ouabain, digitoxin, and the dilactone of pulvic acid*

DRUG	ED <sub>50</sub> $\pm$ s.e.	REL. POTENCY $\pm$ s.e.	POTENCY RATIO	NO. HEARTS
	<i>micrograms per 100 cc.</i>			
Ouabain	37.3 $\pm$ 7.2	—	1	17
Digitoxin	83.8 $\pm$ 15.9	445 $\pm$ 12	1:2:3	21
Dilactone of pulvic acid	44.5 $\pm$ 8.6	836 $\pm$ 22	1:1:2	18

A small number of hearts were analyzed statistically by the method of maximum likelihood (10), and the results are summarized in table 2.

The ED<sub>50</sub> reported in this table must not be confused with the crude and more inexact liminal positive inotropic concentration found in table 1, which was obtained by the preliminary screening technique, using a restricted number of hearts. Rather, the ED<sub>50</sub> represents a fair approximation of the dose necessary to produce a positive inotropic effect in 50 per cent of all hearts tested, and was arrived at by investigating the response of a number of hearts over a broad dosage range.

The high standard errors may be explained on the basis of the small samples of population used. The potency ratio listed in the above table has been calculated from actual concentrations; recalculation on a millimolar basis<sup>4</sup> gives a ratio of 1:18.6 for the ouabain-dilactone of pulvic acid comparison, which agrees well with the crude ratio of 1:21.3 found in table 1.

Other compounds of the unsaturated lactone series which demonstrated a high order of activity were *l*-ascorbic acid, 3-phenyl-3-butene-1,4-diol,

<sup>4</sup> Molecular weight of dehydrated ouabain = 584, of the dilactone = 290.

and 3-pentene-1,4-olide. Furthermore, the 2-pentene-1,4-olide and 3-pentene-1,4-olide showed a cardiotoxic ratio of 8:1 with each other (indicating that the latter was 8 times as potent as the former), which compared favorably with the earlier reported 10:1 cardiotoxicity ratio (1).

In the clavacin series, the high cardiotoxicity of clavacin seemed to be superimposed upon its cardiotonic activity. This effect may be seen in figure 1. Although 10 mgm. per 100 cc. exerted an immediate beneficial effect on the hypodynamic heart, yet within one minute the heart was stopped in systolic standstill, which, however, could be reversed by immediate perfusion with normal Ringer's. Isoclavacin, on the other hand, demonstrated a powerful restorative effect on the hypodynamic heart at the same concentration without toxicity

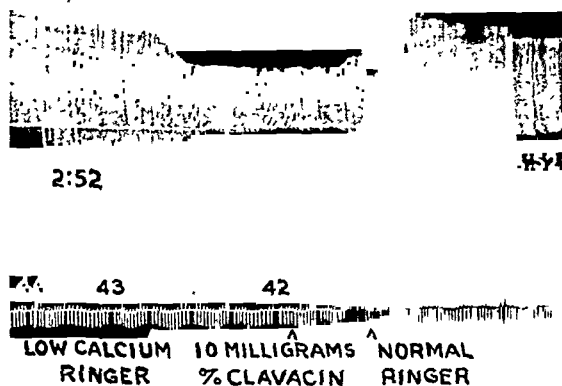


FIG. 1. Cardiotoxicity of Clavacin immediately following its cardiotonic effect. In this and the following figures time of day appears directly below the tracing, while under this may be found heart rate (per minute) and a record of the passage of time in intervals of 5 seconds.

(fig. 2). The methylated derivative, dimethylisoclavacin, showed a comparatively low order of activity.

The unsaturated delta-lactones, coumarin and parasorbic acid, had the least cardiotonic activity of all the unsaturated lactones. Thus, it may be generally stated that an increase in the size of the lactone ring from a five-membered one to a six-membered one results in decreased activity. Coumalinic acid proved to be the most effective of this series, perhaps because of its system of conjugated double bonds. This same situation prevailed in cardiotoxicity experiments reported earlier (1).

2. *Gamma-pyrone series.* The displacement of the carbonyl group from an ortho-orientation with the oxygen bridge to a para-orientation as in the gamma-

pyrones had no diminishing effect upon cardiotonic activity. Meconic acid, for example, had the ability to restore the hypodynamic heart to its maximum contraction at concentrations of 1.25 to 10.0 mgm. per 100 cc. (fig. 3). It was effective, therefore, in the same range of concentration as 3-pentene-1,4-olide. The other gamma-pyrone studied, kojic acid, demonstrated approximately one-tenth this activity.

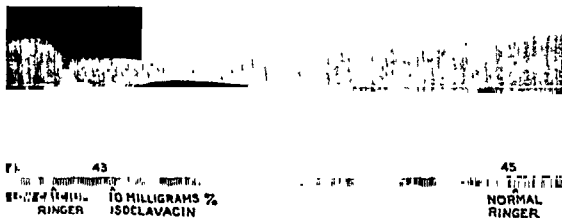


FIG. 2. The cardiotonic activity of Isoclavacin on the hypodynamic isolated frog heart.

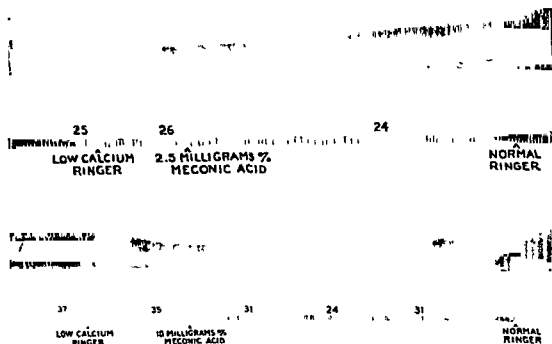


FIG. 3. The cardiotonic activity of Meconic acid on the hypodynamic isolated frog heart—the upper tracing at a level of 2.5 mgm. per 100 cc., and the lower one at 10 mgm. per 100 cc.

3. *Furan series.* All of the agents in this group were devoid of cardiotonic activity. Thus, it may be stated generally that removal of the carbonyl group from the lactone structure leads to a complete loss of cardiotonic potency.

*Mechanism of cardiotonic action.* An insight into the mechanism of the cardiotonic action observed here has been sought in the reports of other investigators and in an accidental observation arising from this work.



1. *Ouabain-lactone interaction.* In the course of experiments in which certain of the lactones and ouabain were tested on the same heart, it became apparent that ouabain was a powerful synergist<sup>5</sup> for these compounds. At a concentration of 10 microgm. per 100 cc. (roughly one-quarter of an  $ED_{50}$ —cf. table 2), ouabain induced recovery in all hypodynamic hearts when it was followed or accompanied by subeffective doses of the lactones in question. Table 3 summarizes these effects.

The fact that the synergism was displayed as well when the ouabain was perfused through the heart before the non-glycosidal compound as when they were perfused together seemed to indicate some fixation of the glycoside in the heart. Stoll has suggested that such fixation may occur through the cholesterol present in the myocardium (11).

TABLE 3

*Synergistic effect of ouabain with various lactones on the hypodynamic frog heart*

COMPOUND	LIMINAL POSITIVE INOTROPIC CONCENTRATION*	LIMINAL POSITIVE INO- TROPIC CONCENTRATION AFTER PERFUSION WITH 10 MICROGM. PER CENT OF OUBAIN
	mgm. %	mgm. %
Ouabain	0.0125	0.0125
2-Keto-3-dimethylaminomethylbutyrolactone	5.0000	2.5000
2-Pentene-1,4-olide	10.0000	5.0000
3-Methyl-5-carboxy-2-pentene-1,4-olide	20.0000	10.0000
Dilactone of pulvinic acid	0.1000	0.0500
3-Pentene-1,4-olide	1.2500	1.0000
3-Phenyl-3-butene-1,4-olide	1.0000	0.6667
Clavacin	5.0000	2.5000
Coumarin	20.0000	10.0000
Coumalinic acid	5.0000	2.0000
Meconic acid	2.5000	2.5000

\* Minimum concentration causing 3 out of 4 hypodynamic hearts to recover to 75% or more of the maximum contraction within 20 minutes.

It was found that the lactone structure was essential for this synergism with ouabain, since the gamma-pyrones failed to show it. Figures 4 and 5 demonstrate these interactions. In these experiments, 10 microgm. per cent of ouabain did not exert a positive inotropic effect within 20 minutes when perfused through the hypodynamic heart alone.

Furthermore, it was interesting to find that the same synergism did not exist between any two of the lactones, nor could any of the lactones synergize the action of ouabain. In other words, if perfusion with any lactone were followed

<sup>5</sup> The term synergist is used here to denote an agent which in itself is cardiotonic, and whose presence lowers the concentration of another cardiotonic agent required to produce a desired effect. No potentiation is inferred, because a potentiator would have no cardiac activity *per se*.

by perfusion with any other lactone (i.e. in sub effective doses) or with ouabain, the positive inotropic action was not seen. Moreover, perfusion with sub-effective doses of any two lactones together failed to show a positive inotropic effect.

In order to determine whether the ouabain lactone synergism were a response to a specific or to a general steroid structure, several experiments were conducted with estrone and androsterone, instead of ouabain, co perfused with sub-effective doses of 3 pentene 1,4 olide. Both of the hormonal substances were devoid of any cardiac activity at a concentration of 100 microgm per 100 cc,

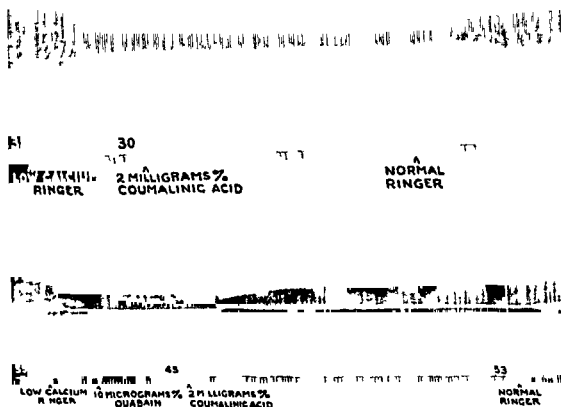


FIG. 4 The synergistic action of ouabain c. The upper tracing demonstrates the failure of effect on an hypodynamic heart at 2 mgm per synergistic interaction.

and in all cases cited, 1 mgm of 3 pentene-1,4 olide per 100 cc had no positive inotropic action. Figure 6 shows the characteristic response observed with estrone. Potentiation with estrone was far more striking than that with androsterone.

**2 Peroxide lactone interaction** Following an early observation of Peugnet (12) that copper was essential for the "beat strengthening" action of l ascorbic acid, Kraye found that the action of this agent could be accounted for by the formation of hydrogen peroxide during the process of dehydrogenation in aqueous solutions (13). Mendez extended this concept to explain the mechanism of cardiotonic action of the angelica lactones (8). It was decided in several of our

experiments to follow this approach indirectly by seeking a potentiation between sub-effective doses of hydrogen peroxide and of the lactones.

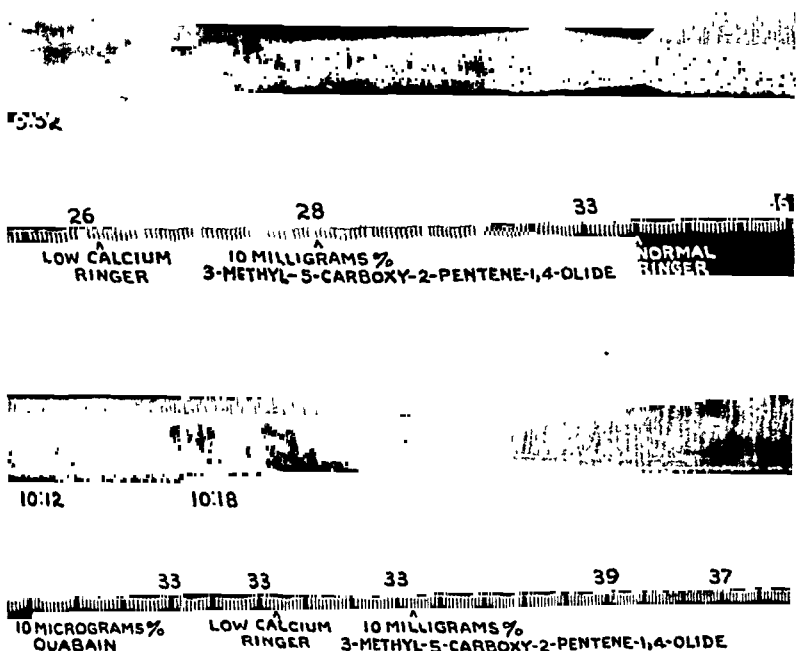


FIG. 5. The synergistic action of ouabain on the cardiotonic effect of 3-methyl-5-carboxy-2-pentene-1,4-olide. The upper tracing demonstrates the failure of the lactone to exert a positive inotropic effect on a hypodynamic heart at 10 mgm. per 100 cc., while the lower tracing shows the synergistic interaction.

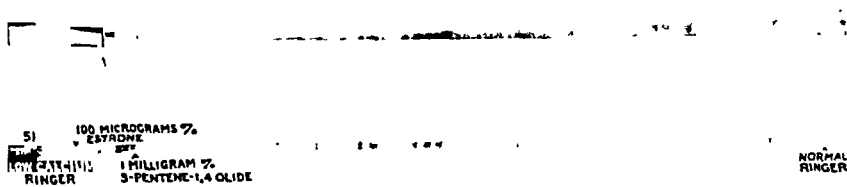


FIG. 6. The interaction of estrone and a sub-effective dose of 3-pentene-1,4-olide on the hypodynamic isolated frog heart.

After it had been established that 10 microgm. of hydrogen peroxide per 100 cc. was devoid of cardiotonic activity (on all hearts observed for two hours), this concentration was chosen for subsequent experimentation. Table 4 contains the results of these experiments.

The most striking effect was seen with ouabain, which showed a cardiotonic

effect at 1 microgm per 100 cc (roughly one fortieth of an  $ED_{50}$ ) when co-perfused with the peroxide (fig 7). It should be noted that ouabain alone at a concentration of one microgm per cent consistently failed to give a positive inotropic response in this work.

The potentiation by peroxide was confined to the lactone series, since neither the gamma-pyrones nor the furans demonstrated the effect. A typical lactone-peroxide interaction may be seen in figure 8 in which a sub effective dose of coumalinic acid when co-perfused with peroxide was capable of partially restoring the hypodynamic heart to its maximum contraction.

The powerful action of the dilactone of pulvic acid was not potentiated in this fashion by hydrogen peroxide, indicating that this agent acts through a different mechanism.

**DISCUSSION** The greater cardiotonic activity of compounds with the delta-2 position of unsaturation provides a close parallel with the greater cardiotonic

TABLE 4

*Interaction of hydrogen peroxide with various lactones on the hypodynamic frog heart\**

COMPOUND	LIMINAL POSITIVE INOTROPIC CONCENTRA TION	LIMINAL POSITIVE INO TROPIC CONCENTRATION WHEN PERFUSED WITH 100 MICROGM PER CENT OF HYDROGEN PEROXIDE
	mgm %	mgm %
Ouabain	0.0125	0.0010
2 Pentene 9,4 olide	10.0000	5.0000
l Ascorbic acid	1.2500	1.0000
3 Pentene 1,4 olide	1.2500	1.0000
Coumarin	20.0000	10.0000
Coumalinic acid	5.0000	2.0000
Dilactone of pulvic acid	0.1000	0.1000

\* Minimum concentration causing 3 out of 4 hypodynamic hearts to recover to 75% or more of the maximum contraction within 20 minutes.

activity of this configuration (1), and adds further evidence to the possibility that this is the position of unsaturation in the lactone residue of such cardiac glycosides as digitoxin and ouabain. This problem, however, is confused by the outstanding activity of the dilactone of pulvic acid, which is composed of two condensed gamma-lactone rings, both of which contain a double bond in the delta 1 position. An explanation for this situation may lie in the relatively high chemical reactivity of the conjugated double bond system, which is present in the dilactone.

From these observations it seems reasonable to infer that while delta 2 unsaturation is most favorable for cardiotonic activity in the simple lactone structure, still the delta 1 position of unsaturation can produce a high (or, perhaps, the highest) order of activity when incorporated in a system of conjugated double bonds.

In an earlier work (1) it was calculated that the  $LD_{50}$  for the dilactone of pul-

vinic acid on the isolated frog heart was  $44.5 \pm 8.6$  microgm. per 100 cc. This value stands out as a major discrepancy when compared with the  $ED_{50}$  for the same compound, calculated in this work as  $86.6 \pm 19.2$  microgm. per 100 cc. Since, however, both doses were arrived at by different experimental maneuvers, they are not strictly comparable. In the case of the toxicity experiments the drug was perfused through the heart after being dissolved in normal Ringer's solution; furthermore, the limiting time in these experiments was 120 minutes. On the other hand, in the determination of cardiotonic activity, the drug was perfused through the hypodynamic heart while dissolved in low-calcium Ringer's;

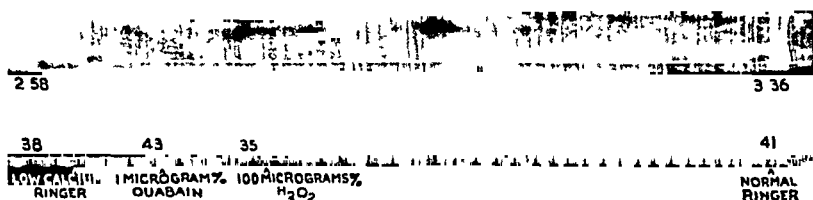


FIG. 7. The potentiating effect of hydrogen peroxide on the cardiotonic action of ouabain.

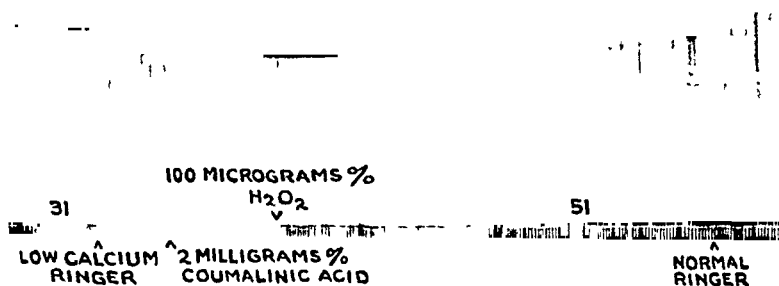


FIG. 8. The potentiating effect of hydrogen peroxide on the cardiotonic action of coumalinic acid.

and the critical time here was 20 minutes. Moreover, it has been shown repeatedly that in the presence of low calcium there is a definite decrease in the toxicity of cardiac drugs for isolated heart preparations (14). The magnitude of discrepancy, however, suggests that, had the time of observation been extended to 120 minutes in the cardiotonic experiments, hearts treated with an  $ED_{50}$  of the dilactone would most probably have died in systolic standstill, despite the interplay of the low-calcium sparing effect. Rather than true cardiotonic effects, therefore, these findings may imply that our observations are reflections of a beat-strengthening action characteristic of the initial toxicity of the chemical agent. In point of fact, moreover, the good correlation between the relative

potencies of ouabain and digitoxin, from both the cardiotoxic (1:2.3) and cardiotonic (1:2.9) points of view, may be interpreted to indicate that their basic mechanism of cardiotoxicity and cardiotonic activity is but an expression of varying degrees of the same chain of chemical events.

The experiments indicating a synergistic action between ouabain and the lactones, and a potentiation by estrone, and to a lesser extent, androsterone on the lactones, may lend support to the suggestion that there is the formation of a steroid-enzyme complex capable of metabolizing a lactone moiety in a manner which yields energy to the contractile mechanism of the heart. In this regard, it has been shown recently that ouabain, digitoxin, and several steroidal substances (cholesterol, digitonin, and sex hormones) are capable of increasing the anaerobic oxidation of lactate by homogenate of heart muscle of vitamin E-deficient guinea pigs (15). To study this mechanism further, it would be desirable to obtain a steroidal compound with an hydroxy group in the C-14 position, a glycoside linked to the C-3 position, and in the C-17 position, a substituent sufficiently active to combine with an unsaturated lactone.

The peroxide effect noted in this work might be looked upon as an extension of some of the earlier observations of Mendez, who reported that the cardiotonic action of certain lactones may be directly ascribed to peroxides formed in solutions in the presence of metallic impurities. This peroxide effect was counteracted by cysteine, glutathione and other —SH compounds, the sulfhydryl group presumably blocking oxidation by complex formation with the metallic impurities (8). Mendez inferred later that the peroxide effect could be mediated by an oxidation process which would interfere with the normal function of sulfhydryl enzymes; such a mediation was demonstrated with certain so-called —SH reagents, such as porphyrindine, iodoacetamide, and mercaptide-forming compounds (16). The potentiation of the lactones by peroxide demonstrated here furnishes further evidence for the importance of an oxidative process in the positive inotropic response of the hypodynamic heart to a cardiotonic drug.

#### CONCLUSIONS

1. Twenty-six lactone antibiotics and synthetic analogs have been studied for their cardiotonic activity on the isolated frog heart made hypodynamic with low calcium Ringers' solution.

2. The dilactone of pulvinic acid exerted a pronounced positive inotropic effect in the same order of concentration as that necessary for the cardiac glycosides, ouabain and digitoxin, to produce the same beneficial effect.

3. All of the unsaturated gamma-lactones displayed some cardiotonic activity. Delta-2 unsaturation appeared more conducive to this effect than delta-1, except in the cases of delta-1 unsaturation associated with a system of conjugated double bonds.

4. Saturation of the lactone structure abolished cardiotonic activity, while alkyl substitution diminished it.

5. Unsaturated delta-lactones were less active than unsaturated gamma-lactones.

6. The gamma-pyrone structure displayed a surprisingly high order of activity.
7. Ouabain demonstrated a synergistic effect with some of the unsaturated gamma-lactones in restoring the hypodynamic frog heart to its maximum ventricular contraction.
8. Hydrogen peroxide potentiated the cardiotonic activity of some of the lactones, with the notable exception of the dilactone of pulvinic acid.

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#### REFERENCES

1. GIARMAN, N. J.: *THIS JOURNAL*, **94**: 232, 1948.
2. CHEN, K. K., AND ELDERFIELD, R. C.: *THIS JOURNAL*, **70**: 338, 1940.
3. CHEN, K. K., CHEN, A. L., AND ANDERSON, R. C.: *J. Am. Pharm. Assoc.*, **25**: 579, 1936.
4. CHEN, K. K., STELDT, F. A., FRIED, J., AND ELDERFIELD, R. C.: *THIS JOURNAL*, **74**: 381, 1942.
5. LINSTEAD, R. P., AND KRAYER, O.: *Science*, **95**: 332, 1942.
6. KRAYER, O., MENDEZ, R., MOISSET DE ESPANES, E., AND LINSTEAD, R. P.: *THIS JOURNAL*, **74**: 372, 1942.
7. KRAYER, O., LINSTEAD, R. P., AND TODD, D.: *THIS JOURNAL*, **77**: 113, 1943.
8. MENDEZ, R.: *THIS JOURNAL*, **81**: 151, 1944.
9. RINKES, I. J.: *Rec. trav. chim. des Pays-Bas*, **49**: 1002, 1930.
10. BLISS, C. I.: *Quart. J. Pharm. and Pharmacol.*, **11**: 192, 1938.
11. STOLL, A.: *The Cardiac Glycosides*, Pharmacological Press, London, 1937, pp. 51-76.
12. PEUGNET, H. B.: *Science*, **90**: 162, 1939.
13. KRAYER, O.: *Proc. Soc. Exper. Biol. and Med.*, **53**: 51, 1943.
14. BAKER, J. B. E.: *Brit. J. Pharmacol.*, **2**: 259, 1947.
15. GOVIER, N. M., YANZ, N., AND GRELLIS, N. E.: *THIS JOURNAL*, **88**: 373, 1946.
16. MENDEZ, R., AND PERALTA, B.: *THIS JOURNAL*, **90**: 128, 1947.

# STUDIES OF THE METABOLISM OF GALLIUM<sup>1</sup>

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In a previous report (1) the findings on the toxic properties of gallium have been reviewed. In order to elucidate in part the mode of action of gallium a study of certain biochemical phases of the problem have been carried out. In this report we give results of the following studies, (a) influence of time on the excretion and retention of injected gallium in the rat and rabbit, (b) influence of time and route of administration on blood gallium, and (c) influence of injected gallium on formed elements of the blood.

**EXPERIMENTAL.** *Influence of time on clearance of gallium in rats.*—The analytical method used in the determination of gallium in tissues and excreta has been given in detail previously (2). It is this method which has been used in the determination of the gallium content of the tissues of rats following a single near-lethal injection of gallium lactate (1, 3). Two or more of these rats were killed at stated intervals from four hours to 180 days following the subcutaneous injection of 100 mgm. Ga/kgm. body weight. Duplicate determinations of gallium content of the liver, kidney and femur from each animal were carried out. In figure 1 are shown the results of the analysis of tissues from rats killed at the indicated intervals. Of particular interest is the rapidity with which gallium enters bone (femur) and is retained there in appreciable quantities for more than 90 days. The liver and kidney while having moderate to high concentrations initially lose much of the gallium by 30 days and are all at a low level after 60 days.

Control animals show no detectable gallium in any tissue with the possible exception of bone. Indications are that normal bone may contain traces of gallium, but of such small magnitude as to be below the threshold of the analytical procedure (<1 ppm).

*Rate and route of excretion of gallium by the rabbit.*—To determine the rate and route of excretion of gallium in the rabbit, four grown albino rabbits were injected with gallium lactate, 90 to 100 mgm. Ga/kgm. body weight. These animals were individually housed in metabolism cages and the urine and feces collected periodically for a period of ten days.

In table 1 are shown the results of studies of the excretion of injected gallium. In these four rabbits the urine accounts for 96 per cent of the gallium excreted. The balance was found in the feces and it seems probable that even this small amount may be due to contamination of the feces by the urine. As will be seen, the gallium rapidly makes its appearance in the urine at a high level, but the drop

<sup>1</sup> The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.



is rapid so that after three days the urinary gallium approaches a constant low level, less than 1 mgm. Ga/day, which continues more than ten days.

In view of the retention exhibited by the rats (fig. 1) the retention by the rabbit of injected gallium is also significant. The total subcutaneous dose of gallium lactate was 90 to 100 mgm. Ga/kgm., and by summation of the total excreted in ten days, we find that more than 85 per cent of the original amount of gallium was fixed in tissues ten days following the injection. This confirms the rat findings and indicates probable marked absorption by the skeletal tissues.

Further study of the gallium content in bone of rats and rabbits has shown that both the soft marrow and periosteum contain approximately the same gallium content as muscle (2 to 6 ppm) whereas the hard structure (cortex) contains 50 to 150 ppm. The trabecular bone (spongy) is still richer, containing from 100 to 300 ppm. This deposition occurs in large part within four hours after injection with gallium lactate and is carried to completion within 24 hours.

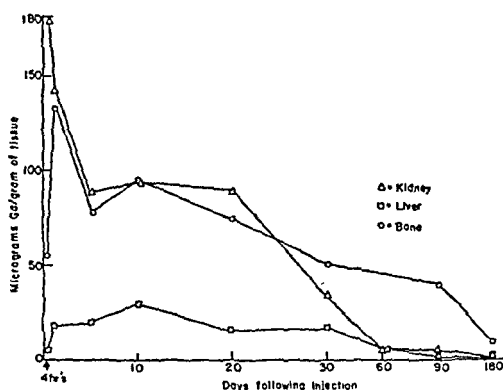


FIG. 1. Ga content of tissues of rats following subcutaneous injection of 100 mgm. Ga/kgm. as gallium lactate.

*Gallium in blood.*—To determine the influence of gallium on the blood, twelve rabbits were injected with gallium lactate, 20 to 35 mgm. Ga/kgm., and bled periodically by heart puncture and the oxalated blood centrifuged. The cells were washed with 0.9 per cent saline, while plasma was analyzed directly for gallium. Analyses of blood plasma from animals injected subcutaneously were compared with results from the same dose of gallium lactate given intravenously in order to determine the influence of mode of administration on plasma gallium.

In figure 2 are presented data derived from studies of rabbits injected intravenously with the indicated dose of gallium lactate. As will be seen whole blood contains considerably less gallium per cc. than does plasma. By washing the cells with 0.9 per cent saline it was found that while no significant amount of gallium is contained in the cells, all is carried by the plasma. As will be seen the ultimate plasma gallium is not affected by increases in dosage, since the fall is quite rapid and at 24 hours the two dosage levels have yielded plasma levels of 1 to 3 microgm. Ga/cc. plasma. These studies were continued for fourteen days at which time the plasma concentration was approaching a trace level (<0.2 microgm./cc.). From two to fourteen days the plasma levels gradually de-

creased from about 2 ppm to the 0.2 ppm level. The points shown on the curves in figure 2 are the average of values obtained from three or more animals. Analyses were run in duplicate.

To determine the influence of route of administration on plasma gallium rabbits were injected with gallium lactate, 20 mgm. Ga/kgm., subcutaneously or intravenously. At intervals thereafter blood was taken by heart puncture and the gallium content of the plasma determined. The intravenous results are shown in Curve B of figure 2. The subcutaneous dosage gave values of 10 to 15 microgm. Ga/cc. plasma one hour after injection. There was no further rise in plasma gallium and the concentration slowly fell to 1-3 microgm. Ga/cc. plasma at 24 hours, identical to the intravenously injected animals. This finding is explained by the slower absorption from the subcutaneous tissues since these tissues, at the site of injection, still contain considerable gallium three days after injection of the lactate.

TABLE 1

*Excretion of gallium following subcutaneous injection of Ga lactate (mgm. gallium excreted during intervals shown)*

RABBIT NO.	TOTAL DOSE OF GA MGm. <sup>1</sup>	EXCRETED IN URINE					EXCRETED IN FECES					% OF TOTAL DOSE EXCRETED IN 10 DAYS
		0-24 hrs	24-48 hrs	48-72 hrs	72-120 hrs	120-240 hrs.	0-24 hrs.	24-48 hrs.	48-72 hrs.	72-120 hrs.	120-240 hrs.	
1	302	19.1 <sup>2</sup>					0.32 <sup>2</sup>					
2	272	25.5		3.0	1.1	4.2	0.06		0.31	0.23	0.09	12.8
3	246	20.9	6.0	2.3	1.4	4.8	0.01	0.13	0.12	0.57	0.23	15.2
4	247	8.6	10.3	3.6	1.8	3.9	0.07	0.11	0.04	1.80	0.77	12.5

<sup>1</sup> Dosage 90-100 mgm. Ga./kgm. body weight.

<sup>2</sup> Death followed this period.

Examination of the slopes of the plasma curves in figure 2 will show that at very high plasma gallium levels the combination of excretion and tissue deposition is quite rapid. The slope of the curves from 15 to 24 hours indicates that there is a second fixation process. Therefore the curves as observed seem to be a resultant of two independent processes, the nature of which we have no knowledge at this time.

*Influence of gallium lactate on formed elements of blood.*—Rabbits, injected with near-lethal, and often lethal quantities of gallium lactate have shown no significant or marked changes in the formed elements of the blood. The rabbits used in these studies were those used in the toxicity determinations and represent a total of twenty animals. These rabbits had received gallium lactate intravenously in excess of 20 mgm. Ga/kgm. or if treated subcutaneously received in excess of 90 mgm. Ga/kgm.

There was often a transitory decrease in the lymphocytes and an increase in segmented cells in 24 to 48 hours after injection, which lasted less than 48 hours. The total white cell count was within normal limits, as were the erythrocytes.

In terminal stages total white cell counts did increase, however this is considered to result from terminal pneumonia since the animals died of respiratory failure, probably caused by paralysis.

**DISCUSSION.** From a study of the data presented in this paper, together with the toxicity, and distribution studies reported previously it seems probable that in gallium we have a new tool for the study of bone metabolism, both in the embryonic and post natal stages. By the analytical procedures developed it is possible to determine micro quantities of gallium, so that administration of non-toxic amounts of gallium lactate will occasion no great change in the metabolic economy and will serve to tag bone structures at different stages of development.

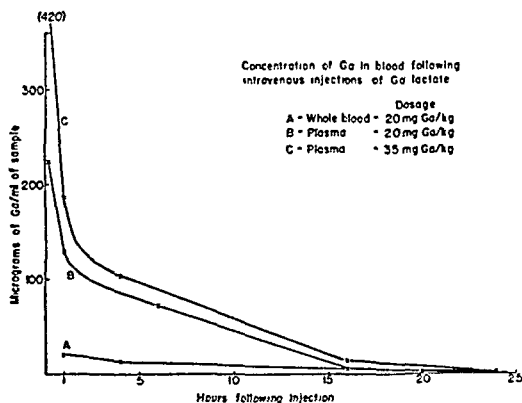


FIG. 2

#### SUMMARY AND CONCLUSIONS

Excretion of injected gallium is via the urine, with greater than 85 per cent of a near toxic dose retained in the rabbit more than ten days.

The liver and kidney fix appreciable quantities of the gallium where it is retained in part for more than 30 days. Gallium enters the bone rapidly, in less than four hours, and is retained in large degree for more than 90 days.

Gallium is transported wholly by the plasma, but is largely cleared therefrom within 24 hours. The influence of the amount of intravenous gallium is transitory since at 24 hours the plasma has attained a constant level of 1 to 3 microgm./cc. The mode of injection influences markedly the initial plasma gallium levels. Intravenously the plasma gallium is initially high and drops rapidly during the first six hours while subcutaneous injection produces only a moderate plasma concentration, which falls slowly to a constant figure by the end of 24 hours.

No significant changes were observed in the formed elements of the blood by injection of fatal or near-fatal amounts of gallium lactate.

It is suggested that gallium may prove to be a new tool for the study of bone metabolism, both in embryonic and post natal stages of growth.

#### REFERENCES

1. DUDLEY, H. C., AND LEVINE, M. D.: THIS JOURNAL, (In Press).
2. DUDLEY, H. C.: THIS JOURNAL, (In Press).
3. DUDLEY, H. C., AND GARZOLI, R. F.: J. Amer. Chem. Soc., 70: 3942, 1948.

# THE EFFECT OF LIVER DAMAGE ON THE ACTIVITY OF G-STROPHANTHIN IN THE RAT

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Under certain conditions the rat can tolerate about a thousand times as much g-strophanthin as the cat (1-4). This resistance of the rat is due to the ability of the rat heart to function in the presence of high concentrations of g-strophanthin (5) and due to the rapid elimination of this glycoside by the rat (1-4). Data were presented previously (6) which demonstrated the importance of the liver for this elimination process. The present study is a continuation of previous work and deals mainly with the effects of hepatotoxic agents (carbon tetrachloride and phosphorus) on the sensitivity of the rat to g-strophanthin. The effects of these agents on the lethal dose, overall elimination rate, hepatic excretion and the sensitivity of the rat heart to g-strophanthin have been studied.

**MATERIALS AND METHODS.** The methods employed were essentially the same as those used in a previous study (6).

The g-strophanthin used was a crystalline material containing 13 per cent water of crystallization. The concentration of g-strophanthin infused was 0.5 per cent in physiological saline. The rates of administration of g-strophanthin varied between 9 and 170 mgm. per kgm. per hour and four to twelve animals were used with each rate of administration. The heart was under direct observation and the infusion was continued until ventricular fibrillation or diastolic standstill occurred. All experiments were performed on male rats anesthetized with Amytal (0.06 to 0.09 gm. per kgm.) given intraperitoneally.

Hepatectomy was performed by eviscerating the amytalized rats (6, 7). In a number of hepatectomized rats the kidney pedicles were ligated and following this procedure the lethal dose of g-strophanthin was determined by the constant infusion method.

Carbon tetrachloride and phosphorus poisoning was produced by injecting these substances subcutaneously daily for three consecutive days. The dosages employed were 0.8 cc. per kgm. of carbon tetrachloride and 0.5 mgm. per kgm. of phosphorus dissolved in olive oil. Twenty-four hours after the last injection of the hepatotoxic agent the rats were anesthetized with Amytal. The lethal dose of g-strophanthin was then determined by the constant infusion method. The treatment of rats with toxic doses of carbon tetrachloride or phosphorus resulted in a 15-25 per cent reduction in body weight. The infusion rates and lethal doses were based on the final weights of these animals. Following completion of the experiment the livers were removed, weighed, fixed and stained with eosin and hematoxylin.

The rate of elimination of g-strophanthin was determined by the method of Hauptstein (8). It consists essentially of the determination of the lethal dose of the glycoside at different rates of administration. In the rat a decrease in infusion rate resulted in an increase in the lethal dose. The elimination rate per kgm. per hour was calculated by dividing the differences between the lethal doses determined at various rates of infusion by the corresponding differences in the time of infusion.

Hepatic excretion was determined by assaying the bile of anesthetized rats collected from the cannulated common bile duct. Collection of bile was started after the single injection of 5 mgm. per kgm. of g-strophanthin and was continued for 45 minutes. The

bile was diluted 1:40 or 1:100 with modified Locke solution and assayed on five to eight isolated frog hearts (7, 8).

Langendorff hearts were prepared from rats under Amytal anesthesia. The chest was opened under artificial respiration, the heart was quickly removed and the aorta cannulated to the perfusion system. The perfusion fluid was Locke's solution (9), kept at a temperature of 37 to 38°C. The perfusion pressure was 90-100 cm. of water and the rate of perfusion was regulated to be about 2 cc. per minute. The heart contractions were recorded on a smoked drum by means of a suitable lever. After a control period of 30 minutes with Locke's solution as perfusion fluid, the heart was perfused with a 1:10,000 solution of g-strophanthin in Locke solution. The time of appearance of auriculoventricular block and systolic standstill was recorded. The hearts were observed for a period of 60 to 90 minutes.

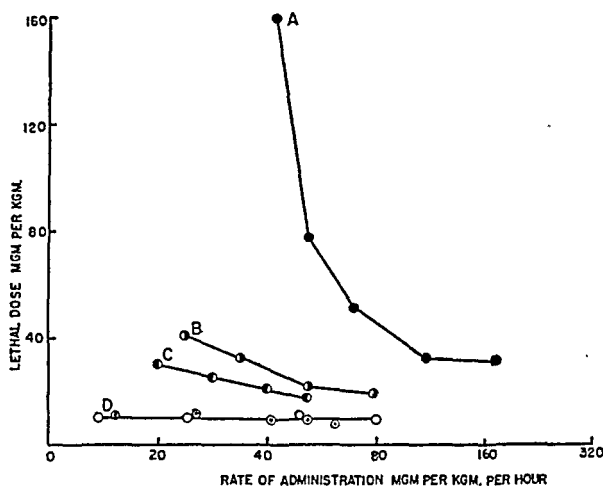


FIG. 1. Effect of rate of administration on the lethal dose of g-strophanthin in the rat under varying conditions. All animals under amytal anesthesia.

- Control rats
- ⊙—⊙ Rats pretreated with carbon tetrachloride
- ⊖—⊖ Rats pretreated with phosphorus
- Eviscerated rats
- ⊙—⊙ Eviscerated rats with kidney pedicles ligated

**RESULTS.** In the rat anesthetized with Amytal a decrease in rate of infusion resulted in a marked increase in the lethal dose of g-strophanthin (fig. 1 curve A). Thus a reduction of the rate of infusion from 170 to 45 mgm. per kgm. per hour resulted in a five- to sixfold increase in the lethal dose. This was due mainly to the elimination of the glycoside by the liver since hepatectomy completely abolished this characteristic increase (fig. 1 curve D) (see also (6)). Following hepatectomy there was no significant increase in the lethal dose when the rate of administration of g-strophanthin was reduced from 54 to 9 mgm. per kgm. per hour.

Pretreatment of the rats with carbon tetrachloride or phosphorus reduced the lethal dose of g-strophanthin at all rates of administration studied (fig. 1 curves B and C). The slope of the curve relating rate of administration and lethal dose is probably an index of the elimination rate. For the normal Amytalized animals

this slope is much greater than for the  $\text{CCl}_4$  or phosphorus treated rats. This reduction in overall elimination has been confirmed further by calculating the elimination rate by Hauptstein's method (8) and the results are given graphically in figure 2. It can be seen that in both the  $\text{CCl}_4$  and phosphorus treated groups elimination has been considerably reduced. Furthermore, it is interesting to note that the elimination rate is determined by the rate of administration; the lower the administration rate the smaller is the rate of elimination per unit of time.

The method of Hauptstein for determining elimination rate is an indirect method and may be open to criticism. We have thus determined the excretion

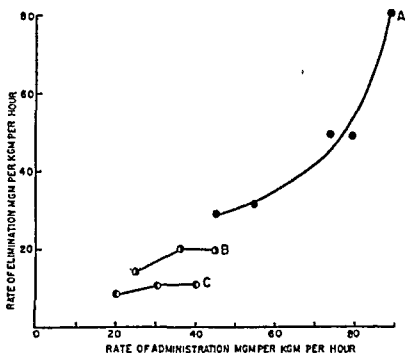


FIG. 2. The elimination of g-strophanthin by the rat determined by the method of Hauptstein (8).

- A. Control rats
- B. Carbon tetrachloride treated rats
- C. Phosphorus treated rats.

of g-strophanthin in the bile of normal,  $\text{CCl}_4$  and phosphorus poisoned anesthetized rats. The bile collected for 45 minutes was assayed on isolated frog hearts for cardiac activity. This assay procedure does not have the accuracy of the standard cat method but has the advantage of being useful in the detections of low concentrations of the glycoside (25–50 microgm. of g-strophanthin). Attempts to apply the alkaline picrate reaction (10) to bile containing g-strophanthin were not successful because of interference of some bile constituents with this reaction. The results of these excretion studies have been summarized in table 1. It can be seen that both  $\text{CCl}_4$  and phosphorus have markedly impaired the hepatic excretion of g-strophanthin. Thus both direct and indirect methods of elimination show that the hepatotoxic agents reduce the elimination rate of this glycoside. The two methods of determining elimination are not quantitatively comparable since the experimental conditions are different for each of the two methods employed.

If the decrease of the lethal dose of g-strophanthin produced by pretreatment with hepatotoxic agents was due to liver impairment only, there should be no significant difference in this lethal dose between hepatectomized normal and hepatectomized rats pretreated with  $\text{CCl}_4$  or phosphorus. Such an experiment was performed and the results obtained are given in table 2. It is clear that the pretreated rats are about twice as sensitive as the untreated animals. This marked difference can be explained only on the basis of some extrahepatic effects of  $\text{CCl}_4$  and phosphorus which increase the sensitivity to g-strophanthin. These extrahepatic effects could be: (a) damage to some extrahepatic elimination mech-

TABLE 1

*The excretion of g-strophanthin in rat bile under varying conditions*

G-strophanthin was injected intravenously in a dose of 5 mgm. per kgm. following which bile was collected for 45 minutes and then assayed on isolated frog hearts (see Methods). Each figure represents the result obtained on one rat.

	G-STROPHANTHIN IN BILE: PER CENT OF TOTAL AMOUNT OF G-STROPHANTHIN INJECTED		
	Control rats	$\text{CCl}_4$ treated rats	Phosphorus treated rats
	70	20	20
	60	40	30
	80	30	
	70	30	
Average.....	70	30	25

TABLE 2

*The effect of carbon tetrachloride and phosphorus poisoning on the lethal dose of g-strophanthin in hepatectomized rats*

	NUMBER OF RATS USED	RATE OF ADMINISTRATION MG. PER KG. PER HOUR	LETHAL DOSE MG. PER KG.	TIME TO PRODUCE CARDIAC ARREST MIN.
Controls.....	10	54.3 $\pm$ 0.6	9.6 $\pm$ 0.5	10.5 $\pm$ 0.6
Phosphorus poisoning.....	6	54.8 $\pm$ 0.6	4.9 $\pm$ 0.4	5.4 $\pm$ 0.6
Carbon tetrachloride poisoning.....	7	55.3 $\pm$ 0.5	4.5 $\pm$ 0.4	4.8 $\pm$ 0.8

anism (b) a change in the extrahepatic distribution of the glycoside and (c) a change in the sensitivity of the heart to the glycoside. Attempts have been made to study these three factors. In their studies Hatcher and Eggleston (3) have pointed out that under certain conditions the urine of rats may contain small quantities of cardiac glycosides. It was thus possible that the hepatotoxic agents had damaged the renal excretion mechanism. We have tested this point by ligating the kidney pedicles in eviscerated rats. The results obtained have been plotted in figure 1 and with our method no significant change in the lethal dose could be produced by this procedure. It is thus improbable that the differ-

ences between the  $\text{CCl}_4$  poisoned and the untreated eviscerated rats could be explained on the basis of kidney damage produced by the hepatotoxic agents.

Changes in extrahepatic binding of g-strophanthin produced by the hepatic poisons could not be determined, since no significant binding of g-strophanthin could be demonstrated by either the kidney, blood serum and striated voluntary or cardiac muscle.

Change in sensitivity of the heart to g-strophanthin was studied on isolated rat hearts obtained from normal,  $\text{CCl}_4$  and phosphorus poisoned animals. The results have been summarized in table 3. It can be seen that the hearts of both the poisoned groups of animals were significantly more sensitive to g-strophanthin than the hearts of normal rats. This sensitizing effect of  $\text{CCl}_4$  and phosphorus pretreatment of the heart probably explains the differences in sensitivity observed in the eviscerated rats. Heubner and Hecht (11) have found a similar sensitization of the isolated guinea pig heart as a result of phosphorus poisoning.

TABLE 3

*The influence of carbon tetrachloride and phosphorus on the sensitivity of rat hearts to g-strophanthin*

Isolated rat hearts, Langendorf technic, perfusion fluid Locke's solution containing 1:10,000 g-strophanthin. Hearts were obtained from normal rats and from carbon tetrachloride and phosphorus poisoned rats anesthetized with Amytal.

	NUMBER OF HEARTS STUDIED	NUMBER OF HEARTS SHOWING BLOCK	NUMBER OF HEARTS SHOWING SYSTOLIC STANDSTILL	NUMBER OF MINUTES TO PRODUCE STANDSTILL
Controls.....	7	1	0	Greater than 60 minutes
Phosphorus poisoning.....	5	3	5	41
Carbon tetrachloride poisoning .....	7	3	6	30

DISCUSSION. In the rat, carbon tetrachloride and phosphorus poisoning produced a marked reduction in the lethal dose and rate of elimination of g-strophanthin. The results presented show that this reduction in lethal dose is due to at least two factors: (a) decrease in the hepatic excretion rate and (b) an increase in the sensitivity of the rat heart to g-strophanthin. The mechanism of this increase of sensitivity of the heart is not known. A third possible factor, namely, changes in extrahepatic distribution, could not be studied because no glycoside could be found in extrahepatic tissue by the extraction procedures employed.

Hepatotoxic agents are frequently employed to prove the hepatic excretion or destruction of a substance. The results presented here indicate that such conclusions are not reliable unless the hepatic mechanism can also be demonstrated by means other than the use of hepatotoxic agents. Changes in a biological effect as a result of the treatment with hepatotoxic agents are not necessarily due only to liver damage. Extrahepatic effects of these poisons must first be excluded before reliable conclusions can be drawn.



## SUMMARY

In the rat surgical hepatectomy (evisceration) reduces the lethal dose and elimination of g-strophanthin. Exclusion of the kidney has no detectable effects on the lethal dose of this glycoside.

Carbon tetrachloride and phosphorus poisoning produces a reduction in the lethal dose of g-strophanthin. This reduction is due to the reduced excretion of g-strophanthin by the liver and to an increase in the sensitivity of the rat heart to g-strophanthin.

## REFERENCES

1. GUNN, J. A.: *THIS JOURNAL*, 4: 225, 1913.
2. STRAUB, W.: *Arch. exper. Pathol. u. Pharmacol.*, 84: 223, 1919.
3. HATCHER, R. A., AND EGGLESTON, C.: *THIS JOURNAL*, 12: 405, 1918-1919.
4. HEUBNER, W., AND NYARY, A. V.: *Arch. exper. Pathol. u. Pharmacol.*, 177: 60, 1935.
5. GENUIT, H.: *Arch. exper. Pathol. u. Pharmacol.*, 188: 285, 1937.
6. FARAH, A.: *THIS JOURNAL*, 86: 248, 1946.
7. RUSSEL, J. A.: *Am. J. Physiol.*, 136: 95, 1942.
8. HAUPTSTEIN, P.: *Arch. exper. Pathol. u. Pharmacol.*, 126: 121, 1927.
9. JACKSON: *Experimental Pharmacology and Materia Medica*, 2nd Edition, page 44, 1939, C. V. Mosby Company, St. Louis.
10. LENDLE, L., AND PUSH, F.: *Arch. exper. Pathol. u. Pharmacol.*, 177: 550, 1935.
11. HEUBNER, W., AND HECHT, G.: *Nachr. Ges. Wiss. Gottingen Math.-physik Kl.* 220, (1926). Quoted from Lendle, L.: *Heffters Handbuch der exper. Pharmacol. Ergänzungswork I*, Page 123.

# FURTHER OBSERVATIONS ON THE EFFECT OF PROLONGED THIOPENTAL (PENTOTHAL) ANESTHESIA ON METABOLISM OF CARBOHYDRATES AND OF PROTEINS IN DOGS<sup>1</sup>

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In 1946 it was reported from this laboratory (1) that prolonged thiopental (Pentothal) anesthesia caused a prominent and long maintained increase in the blood sugar and reduction of the liver glycogen. The evidence suggested that there may be a disturbance of the enzyme systems of the liver during prolonged Pentothal anesthesia, since those animals on inanition diets and those on normal diets prior to anesthesia failed to convert glucose (administered during anesthesia) into glycogen. During prolonged anesthesia there was described a progressive decrease in the removal of bromsulphalein from the serum by the liver. The view was expressed that the liver was likely "taxed" or placed under "strain" by prolonged Pentothal anesthesia, hence the embarrassment of certain of its metabolic processes.

Several questions arose during the work previously reported, which we shall attempt in this paper to answer:

1. What is the effectiveness of insulin in controlling the hyperglycemia and the reduction of liver glycogen during prolonged Pentothal anesthesia?

2. To what extent is it demonstrable that the protein enzyme system (concerned with deamination) like the carbohydrate enzyme system (concerned with the glycogenic-glycogenolytic pathways), is affected by prolonged Pentothal anesthesia?

3. To what extent can the liver be "protected" from "strain" or "taxation" during prolonged Pentothal anesthesia by high carbohydrate and/or high protein diets?

We believe that the answers to these questions are not only important from the point of view of the efficacy of the prolonged use of Pentothal as an anesthetic agent, but also they indicate the importance of the nutritional state during prolonged Pentothal anesthesia, and the possible role of the liver in the metabolism of Pentothal.

**METHODS.** The experiments herein reported were designed to study in dogs: 1) the effects of insulin on the blood sugar level, on the blood lactate and liver glycogen during prolonged Pentothal anesthesia; 2) the effect of prolonged Pentothal anesthesia on the conversion of the nutritional state on the course of prolonged Pentothal anesthesia. All animals were anesthetized with 15-20 mgm./kgm. Pentothal sodium, administered intrave-

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<sup>1</sup> An abstract of this work appeared in the Federation Proceedings, 1947 and was presented before the Federation of the American Societies for Experimental Biology, Pharmacology Section May, 1947. This work was made possible in part by a grant from the Rockefeller Foundation to Howard University School of Medicine.

nously, prior to which blood samples were drawn for the chemical determinations mentioned below. The animals were maintained in condition of surgical anesthesia by administering Pentothal intravenously, when necessary, using the thoracic wall reflex, described by Whitehead and Draper (2), as a means of determining the state of anesthesia. In the experiments where insulin was administered, the initial dose was given at the time the anesthesia was begun and thereafter at intervals of one-half hour (in some instances) and one hour (in other instances) during the experimental period. In experiments where glucose was used, 50 cc. of 50 per cent solution were injected intravenously just prior to the administration of the anesthetic and no more during the course of the experiment. In the amino acid experiments usually 10 mgm. of arginine were injected at the beginning of the anesthetic period and each hour during the experiment. In some few instances 20 to 40 mgm. were injected at the beginning of the experiment and no more during the course of anesthesia. By and large, each animal used in these anesthesia experiments had its own control period in which the effect of insulin on the blood sugar, liver glycogen and blood lactate, and the conversion of amino acid nitrogen to urea were studied in the unanesthetized state. Glucose, insulin and amino acid were administered the same as in the experimental period. Blood samples were drawn hourly.

Animals on high carbohydrate diet received daily 20 gm. of lactose and 20 gm. of corn starch preceding the experimental period for one week in addition to their regular food. They received also during this period 10 cc. of 5 per cent glucose, given intravenously each day. Animals on high protein diet received 20 to 30 gm. of "Amigen" or "Delcos granules" a day for one week in addition to their regular food. Blood sugar was determined on 2 cc. samples of protein-free filtrate, using the Gibson modification of the Folin-Wu method for preparation of the filtrates (3). The manometric method of Hastings and Avery (4) was used for the determination of blood lactate. The amino acid nitrogen was determined by the Folin colorimetric method (5). The urea was determined by the method of Karr (6). The bromsulphalein method was used for studying liver function (7). In each of the above analyses, tubes containing the colored solutions were read in a Klett-Sumerson photo-electric colorimeter.

At the end of most experiments, and in some instances after 12 hours, the liver was removed and weighed, and 30 gm. samples were minced and prepared for digestion and glycogen determination, using the well-known Benedict method.

**RESULTS.** Figure 1 shows the effect of insulin on the blood sugar and the lactic acid during the course of anesthesia. It can be seen here that doses of 4 to 10 units of insulin per half-hour (depending on whether or not and in what amount glucose is administered) are satisfactory to prevent a sustained hyperglycemia. Our average values for liver glycogen for these experiments (ten in each category) were: (1) anesthetized-no sugar-no insulin 0.8 per cent, (2) anesthetized-sugar-no insulin 0.9 per cent, (3) anesthetized-no sugar-insulin 1.4 per cent, and (4) anesthetized-sugar-insulin 2.0 per cent. The unanesthetized control liver glycogen averaged 2.8 per cent. These results show that insulin administered during prolonged Pentothal anesthesia not only controls the blood sugar, but prevents the depletion of the liver glycogen during the course of anesthesia. Probably, in the presence of glucose simultaneously administered insulin facilitates some storage of glycogen.

Figure 2 shows that there is also a disturbance of the intermediary metabolism of proteins. The amino acids administered during anesthesia increase in the blood the first hour and remain increased throughout the anesthetic period, in contradistinction to the control, which rises the first hour, but begins to return toward normal by the second hour. Concomitantly there is a decrease in the

urea formation during anesthesia as compared with the control periods of no anesthesia. There were fifteen experiments in this series.

Figure 3 represents results obtained from a study of the mgm./kgm. and mgm./kgm./hr. of Pentothal necessary to keep animals under surgical anesthesia

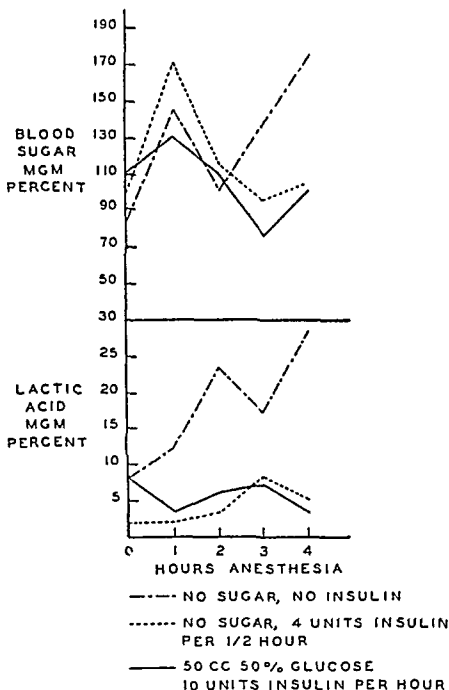


FIG. 1. A typical experiment showing changes of blood sugar and of lactic acid, and the influence of insulin on the same during prolonged Pentothal anesthesia. The first dose of insulin was administered at the beginning of the anesthetic period and subsequent doses each hour during anesthesia. The anesthetized untreated (insulin glucose) animals represent the controls for this experiment.

on normal, high carbohydrate and high protein diets. It can be seen that animals on high protein diets required the greatest amount of Pentothal for hour to hour surgical anesthesia, followed by animals on high carbohydrate diets and then by animals on normal diets. This becomes more significant as we note that animals on high protein diets retained in the serum after 4 hours of anesthesia

only 2 to 3 per cent bromsulphalein ( $\frac{1}{2}$ -hour blood samples); the high carbohydrate group retained 5 to 7 per cent and the normal diet group retained 15 to 20 per cent. Furthermore, the glycogen values of the liver in the high protein group averaged 3.4 per cent; those of the high carbohydrate group averaged 2.0 per cent, and those of the normal diet group averaged 0.8 per cent.

**DISCUSSION.** Our results suggest that there is indication for the administration of insulin during prolonged Pentothal anesthesia, for not only is the blood

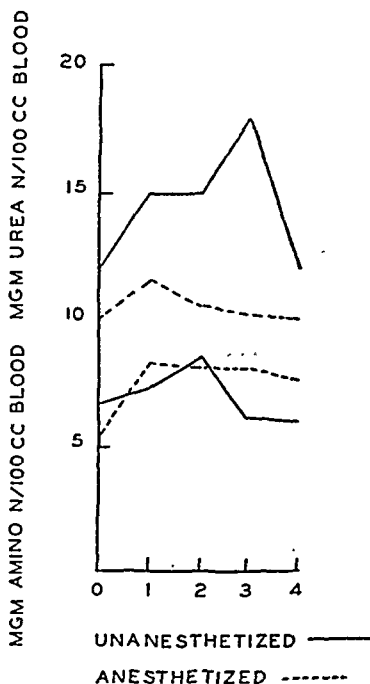


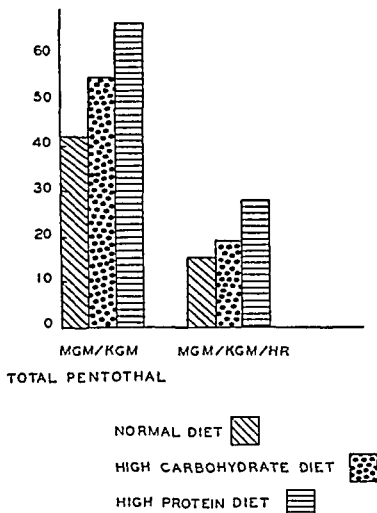
FIG. 2. A typical experiment showing the changes in the blood amino acid nitrogen (lower two curves) and the urea nitrogen (upper two curves) during the course of anesthesia. The unanesthetized period represents the control period.

sugar controlled, but more important than that is the fact that pathways of intermediary carbohydrate metabolism are apparently undisturbed and glycogen depletion is inhibited. In a previous communication, mentioned above, we called attention to the possibility that prolonged Pentothal anesthesia interferes with the glycogenic-glycogenolytic pathway.

Our evidence suggests that the conversion of amino acid nitrogen into urea is also affected by prolonged Pentothal anesthesia. Obviously the liver is not handling excess quantities of amino acids as well in the anesthetized as compared with the unanesthetized state. The increases in blood amino acids correspond to a decrease in the production of urea.

The fact that animals on high protein diets require a greater quantity of Pentothal to maintain surgical anesthesia is to us significant, and it is more significant that animals on high protein diets show less bromsulphalein in their serum after

4 hours of anesthesia than animals on high carbohydrate or on normal diets; and that the glycogen stores show the same relationships. Indeed it seems to show that protein "protects" the liver best against "taxation" or "stress" that occurs during prolonged Pentothal anesthesia. And, more than this, since "protection" of the liver seems to afford more rapid disposal of Pentothal, it seems to be indirect evidence that Pentothal is handled largely by the liver (probably destroyed). This observation finds strength in the work of Kelly, Adams and Shideman (8) who have recently shown that the duration of Pentothal anesthesia is markedly increased in Eck fistula rats or in rats whose livers had been damaged



1. body weight and the mgm./kgm./four hours when they were on normal diet. Ten animals were used in each group.

by carbon tetrachloride or in subtotally hepatectomized rats. No less significant than the above observations, however, is the point that the course of Pentothal anesthesia is markedly influenced by the nutritional state; particularly do we refer here to a positive nitrogen balance, such as is afforded by a diet high in protein. Burstein (9) has called attention to this recently in his work on the nitrogen balance in relation to anesthesia. He showed that in Pentothal anesthesia, particularly, rats on deficient protein diets detoxify Pentothal much less readily than animals on adequate protein intake. This results in a decrease in the amount of Pentothal necessary to maintain anesthesia and in an increase in the duration of anesthesia in the presence of hypoproteinemia.

## SUMMARY AND CONCLUSION

1. Experiments have been described pointing to embarrassment of the intermediary metabolism of carbohydrates and of protein during prolonged Pentothal anesthesia, and the relation of protein, carbohydrate and normal diets to the course of Pentothal anesthesia.

2. The evidence suggests that insulin is indicated during prolonged Pentothal anesthesia since it not only controls the blood sugar level, but the blood lactate as well, and prevents an extensive depletion of liver glycogen. It is probable that during anesthesia insulin facilitates some glycogen storage when glucose is administered.

3. The evidence seems to show that not only is the carbohydrate metabolism disturbed in prolonged Pentothal anesthesia, but also the protein metabolism particularly the conversion of amino acid nitrogen to urea.

4. The evidence seems to support the view that protein "protects" the liver better than carbohydrate since animals on high protein diet showed higher quantities of glycogen in the liver and disposed of bromsulphalein more efficiently than animals on high carbohydrate or on normal diets.

5. The "protection" of the liver by protein and the rapid disposal of Pentothal by animals on high protein diets seem to be related and these two points may well give indirect evidence that Pentothal is handled largely at the liver (probably by destruction).

6. The nutritional state of the organism, particularly positive nitrogen balance influences the course of Pentothal anesthesia and detoxification of Pentothal.

ACKNOWLEDGMENT. The authors desire to express their appreciation to the Department of Biochemistry, Howard University, for the use of their laboratory in certain phases of this work, and to Mrs. Marianna B. Sewell for the preparation of the figures.

## REFERENCES

1. BOOKER, W. M.: *Anesthesiol.*, 7: 405, 1946.
2. WHITEHEAD, R. W., AND DRAPER, W. B.: *Anesthesiol.*, 8: 2, 1947.
3. GIBSON, R. B.: *Proc. Soc. Exper. Biol. & Med.*, 27: 480, 1930.
4. HASTINGS, A. B., AND AVERY, B. F.: *Peters & Van Slyke, Quantitative Clinical Chemistry*, Vol. II, 1932.
5. FOLIN, O.: *Peters & Van Slyke, Quantitative Clinical Chemistry*, Vol. II, 1932.
6. KARR, W. G.: *J. Lab. & Clin. Med.*, 9: 329, 1924.
7. ROSENTHAL, S. M.: *THIS JOURNAL*, 24: 265, 1924.
8. KELLY, A. R., ADAMS, B. J., AND SHIDEMAN, F. E.: *Fed. Proc.*, 6: 344, 1947.
9. BURSTEIN, C.: Presented before the Congress of Anesthetists, New York, September, 1947.

# THE PHARMACOLOGY OF 3-(N-PIPERIDYL)-1-PHENYL-1-CYCLO- HEXYL-1-PROPANOL HCL (ARTANE) AND RELATED COMPOUNDS

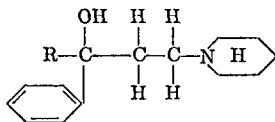
## NEW ANTISPASMODIC AGENTS

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Although most of the compounds in use as antispasmodics are esters, other chemical structures have been shown to possess this property (1-5). In a routine screening program compounds with the structure



were found to possess strong antispasmodic activity when tested upon isolated rabbit ileum. More than one hundred related piperidyl compounds (6-9) were prepared and tested, and compared with the established antispasmodics, *trasentin* and *atropine sulfate* in their ability to relax both normal and spastic segments of rabbit intestine. Of all the variations the one common denominator for high antispasmodic activity was the structure 3-(N-piperidyl)-1-phenyl-1-propanol with substituents on carbon atom number one. Other investigators (10-15) also have demonstrated the relaxing effects of substituted piperidines upon smooth muscle but have failed to recognize the efficacy of the structure which has been shown above. Six of the most active compounds were investigated more extensively with elaboration of the examination of pharmacodynamic action and studies of chronic toxicity.

The chemical names, formulae and code numbers by which these compounds will be designated in this report appear in table 1. All doses are in mgm. per kgm. of body weight unless otherwise specified and comparisons are in terms of dose-action relationship.

**RESULTS. Acute Toxicity.** Table 1 summarizes the data from 1211 mice and 633 rats used in establishing the intravenous and intraperitoneal  $LD_{50}$  for each of the piperidyls. Results with *trasentin* and *atropine sulfate* have been included for reference. Intravenously, the  $LD_{50}$  for the six piperidyls ranged between 31 and 49 mgm. per kgm. in mice and between 25 and 49 mgm. per kgm. in rats. Although these values are of the same order of magnitude it is

<sup>1</sup> Present address: Armour Research Foundation, Chicago, Illinois.







interesting to note that in both species the most toxic compound was 267C, the least toxic 273C and that the 19/20 fiducial limits for these extremes did not overlap. Differences between the other members of the series were less well defined. The intravenous toxicities for the piperidyls may be compared with similar dose effects for 27 and 32 mgm. per kgm. of trasentin and 68 and 41 mgm. per kgm. of atropine sulfate in mice and rats, respectively. Comparable data for trasentin in mice and rats were not found in the literature but Johnson and Reynolds (18) reported that in rabbits the intravenous  $LD_{50}$  for this antispasmodic was 22.5 mgm. per kgm. Lee, Scott, and Chen (19) obtained an intravenous  $LD_{50}$  of 77.5 mgm. per kgm. ( $\pm 3.5$ ) for atropine sulfate in mice, a value which was duplicated satisfactorily in table 1. These data warrant the conclusion that in mice and rats the differences between the intravenous toxicities of the six piperidyls, trasentin and atropine sulfate are of a minor order.

In mice the values for the intraperitoneal  $LD_{50}$  in the piperidyl series ranged from 3.1 to 4.4 times the intravenous values. The corresponding  $\frac{\text{intraperitoneal}}{\text{intravenous}}$  ratios for trasentin and atropine sulfate were 8.1 and 3.1, respectively. Similar ratios in rats ranged from 2.5 to 4.3 for the piperidyls except 275C for which the value was 6.5. The ratio for trasentin was 7.8 and for atropine sulfate, 6.8. Intraperitoneally, 275C was the least toxic of the piperidyls in both mice and rats. Although the high  $\frac{\text{intraperitoneal}}{\text{intravenous}}$  ratios for trasentin in both species and for atropine sulfate in rats may be related to the intraperitoneal hydrolysis of these esters, a similar assumption does not explain the high value for 275C in rats. Perhaps a more important factor, common to all of the high ratios, is a favorable balance between the rates of absorption and detoxication or excretion. The mouse intraperitoneal  $LD_{50}$  of 220 mgm. per kgm. for trasentin and that of 215 mgm. per kgm. for atropine sulfate (table 1) agree well with the value of 240 mgm. per kgm. recorded by Burtner and Cusic (20, 21) for both of these drugs.

No attempt was made to determine the lethal dose in larger animals but the effects of sublethal oral doses were studied. The compounds were administered orally to fasted dogs in doses of 5, 10, 20 and 40 mgm. per kgm. The doses and number of dogs for each compound were: 5 mgm./kgm., 2 dogs; 10 mgm./kgm., 2 dogs; 20 mgm./kgm., 6 dogs; and at 40 mgm./kgm., 4 dogs each for 267C, 274C and 275C. The most noticeable symptoms produced by all the piperidyls except 275C were those which may be attributed to excitation of the central nervous system: restlessness, muscular tremors, myoclonic jerks of the head and neck, and severe clonic and tonic convulsions with opisthotonos.

At doses of 5 and 10 mgm./kgm. 267C and 275C produced no symptoms. At 5 mgm./kgm. 237C, 273C and 274C caused a mild disturbance of equilibrium. At 10 mgm./kgm. these three compounds and also 238C disturbed the equilibrium and in addition produced some muscular tremors and an occasional myoclonic jerk of the muscles of the head and neck.

Of the six dogs given 20 mgm. of 267C, one showed a disturbed equilibrium

and one some muscular tremors. This dose of 275C produced in one dog a disturbance of equilibrium and in four, drowsiness. Twenty mgm. per kgm. of compounds 237C, 238C, 273C and 274C exaggerated the effects observed after lower doses. The severity of the myoclonic jerks and muscular tremors produced by 237C, 238C and 273C indicated that this dose represented the maximal tolerated limit for these compounds.

Forty mgm. per kgm. of 275C produced no muscular tremors or convulsive movements. Equilibrium was disturbed in half of the group and three-fourths of the animals showed drowsiness. Both 267C and 274C at this dose produced muscular tremors. Two dogs given 267C and one given 274C had severe convulsions with opisthotonos and clonic movements.

The results obtained with 275C in dogs must not be interpreted as an indication that this compound is not a convulsant in higher doses. In mice and in rats convulsions always accompanied the administration of doses immediately below and within the lethal range. However, even with fatal doses the convulsions of the animals given 275C were less intense than those produced by the other piperidyls and these animals could be distinguished easily by their low muscular tone.

Sodium pentobarbital diminished the incidence and intensity of the convulsions in rats produced by all of the piperidyls and decreased the mortality except in the group given 275C.

**CHRONIC TOXICITY. Doses.** Multiple doses of these compounds were administered orally to dogs once daily, five days per week for fifteen weeks, and orally or intraperitoneally on the same schedule to mice, rats, guinea pigs, and rabbits for twelve to sixteen weeks. Dosing details are given in table 2.

Changes in hematology, blood pressure, blood sugar, reproductive ability, liver function and kidney function supplemented changes in general appearance, rate of growth and mortality as criteria in the evaluation of chronic toxicity.

**Mortality.** There were no differences in mortality or general appearance between the dosed and control groups.

**Growth.** Average growth curves for rats on each of the compounds and for mice and rabbits on 275C have been included in figure 1. The data represented in this figure show that in rats and mice the difference in weight between the dosed and control groups was never more than ten per cent. All of these compounds were somewhat irritating when injected intraperitoneally; compound 275C was the most irritating member of the series. Rabbits dosed with this compound had numerous abdominal adhesions when autopsies were performed at the end of the period; these adhesions undoubtedly contributed to the difference of thirty per cent between the weights of the dosed and control groups, figure 1. After oral administration no evidence of irritation was observed in any species with any of the compounds.

With a single exception the growth of dogs did not appear modified by daily doses over a period of fifteen weeks; the weights of adults remained static and young animals continued to gain. The exception lay in the group treated with

238C. Two dogs in this group developed such irritability that one was dropped from the series and the other lost nineteen per cent of its starting weight.

*Hematology.* Observations on the hematology of mice, rats, guinea pigs, rabbits and dogs showed no evidence of changes in hemoglobin or total and differential cell counts. Blood from each species was examined for the presence of methemoglobin but none was found.

TABLE 2  
*Schedule for multiple doses\**

COMPOUND NUMBER	ROUTE OF ADMINISTRATION	NUMBER OF ANIMALS†	SPECIES	DAILY DOSE  mgm./kgm.
237C	Oral‡	4	Dog	20
237C	Intraperitoneal	20	Rat	50
237C	Intraperitoneal	10	Mouse	50
237C	Intraperitoneal	8	Guinea pig	50
238C	Oral‡	4	Dog	20
238C	Intraperitoneal	20	Rat	40
238C	Intraperitoneal	10	Mouse	40
238C	Intraperitoneal	8	Guinea pig	40
267C	Oral‡	4	Dog	20
267C	Intraperitoneal	20	Rat	40
267C	Intraperitoneal	10	Mouse	40
267C	Intraperitoneal	8	Guinea pig	40
273C	Oral‡	4	Dog	20
273C	Intraperitoneal	20	Rat	50
273C	Intraperitoneal	10	Mouse	50
273C	Intraperitoneal	8	Guinea pig	50
274C	Oral‡	4	Dog	20
274C	Intraperitoneal	20	Rat	35
274C	Intraperitoneal	10	Mouse	35
274C	Intraperitoneal	8	Guinea pig	35
275C	Oral‡	4	Dog	20
275C	Oral	15	Rat	100
275C	Oral	10	Mouse	100
275C	Intraperitoneal	20	Rat	10
275C	Intraperitoneal	15	Rabbit	30

\* Appropriate controls were included for each species and each compound.

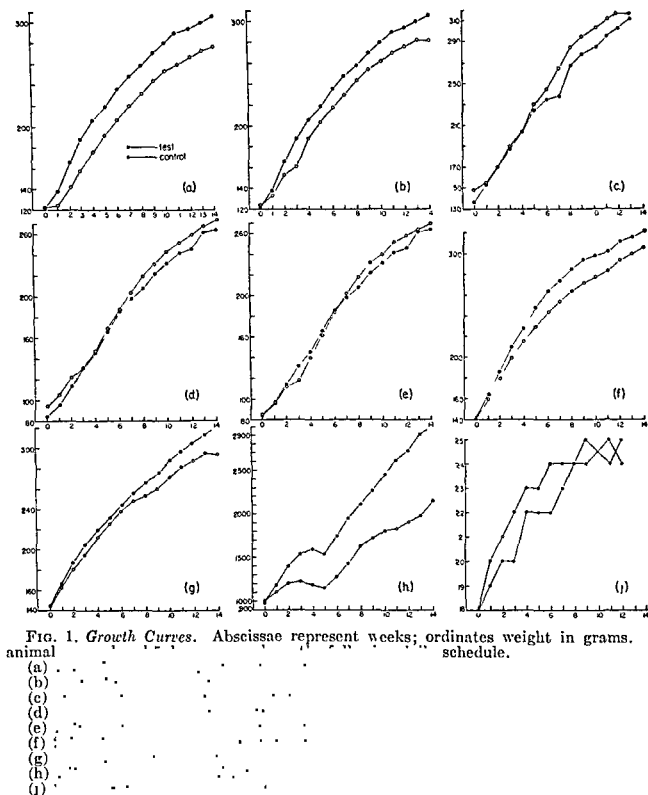
† Dogs were from both sexes; all other animals were males.

‡ Post cibum.

*Miscellaneous Tests.* Prolonged administration caused no significant changes in blood pressure of rats or dogs. Blood sugar levels in rats and phenol-sulfonphthalein tests for kidney function and bromsulphalein tests for liver function in dogs had not departed from the control values after three months of treatment. In a parallel experiment using both male and female rats multiple doses of the piperidyls did not modify fertility.

Robbins (22) has demonstrated the susceptibility of the chick to production of cataract with such agents as 2, 4-dinitrophenol. To determine the effect

of this series of piperidyls on the crystalline lens, twenty-eight 10-day old chicks were divided into seven groups of four each. The controls received 0.25 per cent 2, 4-dinitrophenol in the diet and each of the other groups was given one of the compounds in the same concentration. The controls developed tem-



porary opacity of the lens in four hours and permanent opacity after eighteen to twenty-four hours. After two weeks on the diets containing piperidyls no instance of damage to the eye occurred. Growth was normal.

*Pathologist's Report.* At termination of the periods of multiple dosing animals from the six species studied were sacrificed and subjected to critical gross

and microscopic examination<sup>2</sup>. The pathologist noted abdominal adhesions in the groups dosed intraperitoneally as the only abnormality. This finding was most frequent with 275C.

**SPASMOLYTIC ACTION. Isolated Tissue.** Spasmolytic action of this group of compounds on both normal and spastic segments of rabbit intestine was determined by the method of Magnus and recorded kymographically. The compounds were added directly to 100 cc. of Tyrode's solution in a bath so constructed that the solution could be changed without disturbing the tissue. Temperature was maintained at 38.5°C. Spasms of a myogenic nature were produced by adding 25 mgm. (1:4,000) of barium chloride to the bath and those of neurotropic origin by adding 0.1 mgm. (1:1,000,000) of furfuryl trimethylammonium iodide (furmethide). This compound as shown by Fellows (23, 24) is a powerful parasympathomimetic agent, stable and consistent in its action and capable of producing a maximal spasm lasting many hours.

The relaxant effectiveness of the series has been compared with that of trasentin and atropine sulfate in concentrations of 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.1 and 1.0 mgm. per 100 cc. of Tyrode's solution. Each compound received a minimum of six trials in each concentration against both spastic agents.

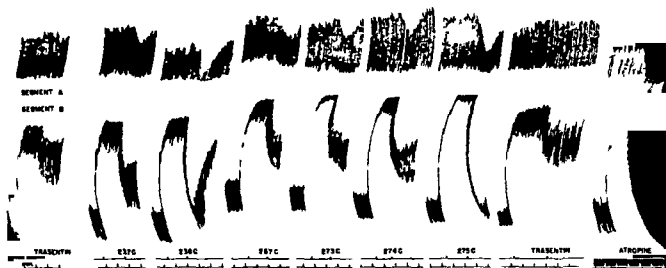
A typical record of one of these experiments is reproduced in figure 2 in which the spasmogenic effect of furmethide was counteracted by 0.005 mgm. of the spasmolytic agent. The effect of this concentration upon activity of the normal intestinal segment is also included. The upward shift of the lower tracing represents the increase in muscle tonus caused by the spastic agent and the downward shift represents the decrease in tonus or the antispasmodic effect. These changes in tonus were measured upon the tracing and the antispasmodic effectiveness estimated in per cent.

Examination of the data taken in more than 500 individual tests, in which the concentrations required to produce similar antispasmodic effects were compared, shows that 275C was one-half, 238C was three-eighths, 274C was one-fifth and 267C, 273C and 237C were each one-eighth as effective as atropine sulfate. It should be mentioned that even though the spasm had been inhibited completely by these compounds normal rhythmical activity of the segment continued, whereas atropine sulfate in sufficient concentration to abolish the spasm usually caused cessation of rhythmical contractions.

When barium chloride was used as the spastic agent the minimal effective concentrations of the antispasmodics were somewhat greater than those required to counteract neurogenic spasms, and more variations were encountered. Tracings from a typical experiment are reproduced in figure 3 in which the spasm induced by barium is antagonized by 0.1 mgm. of the antispasmodic. Testing all the compounds upon a single strip of muscle was not always practicable because of the toxicity of barium. Examination of the data collected in more than 500 tests revealed that the piperidyl derivatives were comparable with atropine sulfate in their capacity to inhibit spasms induced by barium.

**Thiry-Vella Fistulae.** Activity of these compounds upon intestinal loops *in situ* was studied in trained unanesthetized dogs with Thiry-Vella fistulae. Contractions of the loop of intestine were registered upon a kymograph by means of a suitable balloon and

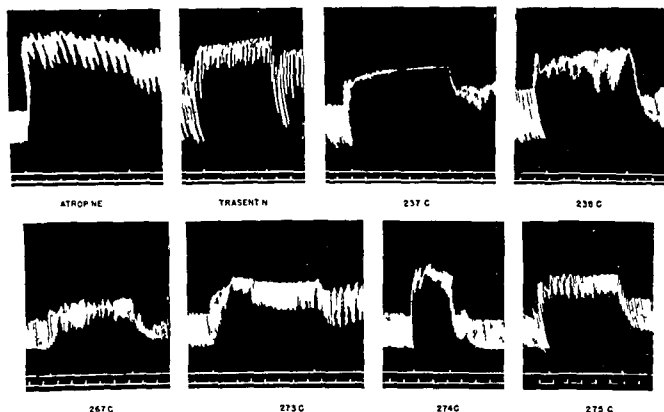
<sup>2</sup> We are indebted to Dr. F. I. Dessau for the gross examinations and histological studies.



COMPARATIVE ANTISPASMODIC ACTIVITY ON ISOLATED RABBIT INTESTINE  
Spasticity Induced by Furfmethide

FIG 2 Record of activity of normal intestinal strip (segment A) and of spastic strip (segment B) in 100 cc bath of Tyrode's solution at a temperature of 38.5°C. Both strips were washed with fresh Tyrode's solution between tests.

At the first signal 0.1 mgm of furfuryl trimethylammonium iodide was added to produce spasm in segment B. At the second signal 0.005 mgm of the antispasmodic agent was added to both normal and spastic strips.



COMPARATIVE ANTISPASMODIC ACTIVITY ON ISOLATED RABBIT INTESTINE  
Spasticity Induced by Barium Chloride

FIG 3 As in figure 2 these intestinal strips were immersed in 100 cc bath of Tyrode's solution at 38.5°C. At the first signal 25.0 mgm of barium chloride was added to induce spasm. At the second signal 0.1 mgm of the antispasmodic agent was added.

pressure recording arrangement (25). Effectiveness was rated upon the ability of the compound to prevent a spasm when administered three minutes prior to the intravenous injection of 0.025 mgm per kgm of furfmethide. This dose of furfmethide causes a severe, usually maximal spasm lasting one or two minutes in this preparation.



The usual procedure in these experiments was as follows: (1) with the recording apparatus in place the animal was allowed time to become adjusted to the surroundings and a tracing was made of the normal activity of the intestinal loop; (2) the standard dose of furmethide (0.025 mgm. per kgm.) was given intravenously and the resulting spasm recorded; (3) when the intestinal tonus was again normal as indicated on the continuous tracing, the test dose of the antispasmodic compound was given, also by the intravenous route, and followed after three minutes by the challenging dose of furmethide.

Antispasmodic effectiveness was estimated in per cent after measuring upon the tracing the extent of the spasm caused by furmethide before and after the administration of the test compound. The animal was challenged with the standard dose of furmethide at ten-minute intervals to determine the duration of the protective action of the antispasmodic. This procedure was repeated in the same dog when practical, and in other dogs using increasing doses of the spasmolytic agent until the spastic action of furmethide was completely blocked, or until symptoms of toxicity from the antispasmodic were observed. Two to four animals were used to determine the effectiveness of each dose of each of the compounds.

The necessity for using essentially asymptomatic doses and the inherent variabilities in the performance of even a well-trained animal do not permit a thoroughly quantitative evaluation; however, figure 4, which summarizes the results obtained in approximately one hundred trials in twenty dogs, shows certain definite differences in the antispasmodic effectiveness of the six piperidyl derivatives and atropine sulfate. It may be seen that 275C was the most effective of the piperidyl group tested in the Thiry-Vella preparation and was the only one which was capable of completely inhibiting the spasmogenic dose of furmethide. Atropine sulfate gave complete protection against the spastic agent at about one-sixth of the dose of 275C required for similar protection. Concurrent observations in these experiments showed that the action of atropine sulfate persisted for periods of 60 to 90 minutes but the effectiveness of 275C usually disappeared in 20 to 30 minutes. Although 267C was the weakest antispasmodic in the piperidyl series its action was dependable and proportional to the dose. This compound was one-fourth as effective as 275C.

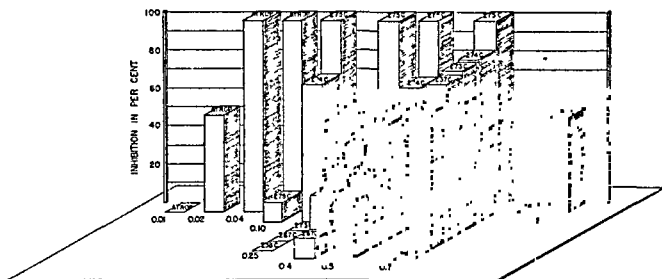
**OTHER EFFECTS.** *Central Nervous System Effects* were chiefly those of stimulation and have been discussed in relation to acute toxicity.

*Mydriasis.* One per cent solution of 275C applied to the conjunctival sac of cats produced mydriatic action almost equal in intensity to that caused by the same concentration of atropine sulfate. After four hours the effect grew less and after 30 hours had almost disappeared while the action of atropine sulfate persisted strongly for more than 168 hours. The other five compounds showed positive mydriatic activity but the action was of a much lower order than that of 275C both in intensity and duration.

In contrast to the duration of its effect when applied locally, 275C when injected intraperitoneally in cats produced mydriasis which outlasted that of atropine sulfate. By this route 1 mgm. per kgm. of 275C produced a mydriasis equivalent in intensity to that given by 0.4 mgm. per kgm. of atropine sulfate. Compounds 238C and 274C produced a positive action but of a lower order than that of 275C. Compounds 237C, 267C and 273C in doses of 1 and 2 mgm. per kgm. produced barely perceptible pupillary changes.

*Sialoschesis.* This effect was determined in etherized dogs by measuring the

flow from Wharton's duct when the submaxillary gland was excited at 30-minute intervals by the intravenous injection of 0.025 mgm. per kgm. of furmethide. Saliva was collected and measured for a period of ten minutes after the stimulation, although control tests indicated that the effect was nearly always complete in five minutes. All of the six compounds tested exerted an atropine-like inhibition of salivary flow. In figure 5 the data comparing this sialoschesis with that of atropine sulfate have been plotted. Curves for only the weakest, 267C in a dose of 0.5 mgm. per kgm. and the most powerful compound, 275C in a dose of 0.25 mgm. per kgm., are shown and compared with atropine sulfate in doses of 0.01 and 0.025 mgm. per kgm. On a dose relationship the activity of atropine sulfate is about ten times that of 275C and a hundred times that of 267C. Compound 238C is one-third as effective as 275C, and 237C, 273C and 274C closely approach the activity of 267C.



COMPARATIVE ANTISPASMODIC ACTIVITY ON THIRY-VELLA LOOPS IN DOGS  
Spasticity Induced by Furmethide

FIG. 4. The compounds were administered intravenously 3 minutes before the intra-  
onium iodide. The dose  
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*Vagal Activity.* Another atropine-like feature of these compounds is their action upon the cardio-vagal mechanism. This effect was reflected in the changes in the carotid blood pressure caused by electrical stimulation of the right vagus nerve in etherized dogs and rabbits. The stimulus employed was of such strength as would produce a fall of approximately 30 mm. of mercury. At ten- to fifteen-minute intervals after intravenous administration of the compound the right vagus was stimulated and the fall of the blood pressure compared with the effect of the same procedure during the pre-dosing period. Compound 275C was about one-tenth as active as atropine sulfate in the dog (figure 6) since 0.5 mgm. per kgm. caused inhibition of vagal activity of about the same intensity and duration as 0.05 mgm. per kgm. of atropine sulfate. Compounds 237C and 274C, also shown in figure 6, displayed the weakest vagal inhibitory action; compounds 238C, 273C and 267C, not shown in figure 6, occupied intermediate positions.

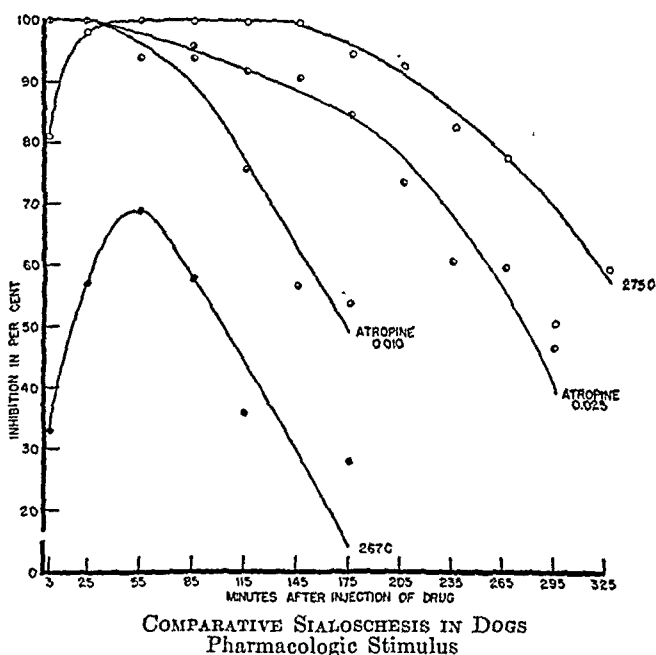


FIG. 5. Saliva was collected from Wharton's duct. Stimulation was produced by the intravenous injection of 0.025 mgm. per kgm. of furfuryl trimethylammonium iodide before and at the recorded intervals after the intravenous administration of the antispasmodic. The doses were: 267C, 0.5 mgm./kgm.; 275C, 0.25 mgm./kgm.; atropine sulfate, 0.010 and 0.025 mgm./kgm.

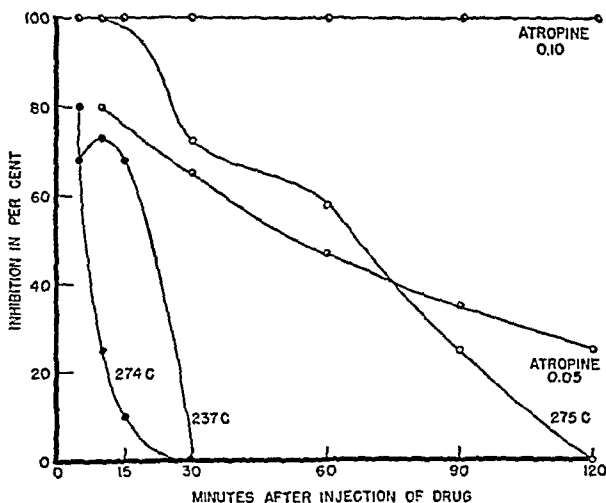


FIG. 6. Vagal inhibition was in relation to the prevention of a fall in blood pressure when the right vagus was stimulated electrically at intervals after one intravenous injection of the compound. The doses were: 275C, 237C and 274C, 0.5 mgm./kgm.; atropine sulfate, 0.05 and 0.10 mgm./kgm.

*Blood Pressure and Respiratory Effects.* All of the members of this series caused a transient fall in the blood pressure following intravenous injection in etherized dogs. The action was less than that of atropine sulfate given in the same doses and the pressure returned to normal in about one minute. The greatest depressor activity was shown by 237C and the least by 267C and 274C. A dose of 10 mgm. per kgm. of any of these compounds if injected rapidly in etherized dogs may cause death. Respiration was unaffected until a fatal dose had been given and it was difficult to determine whether circulation or respiration was first to fail.

*Diuretic Action.* By the Lipschitz assay (26) in rats the following values for diuretic potency were obtained: urea, 1; theophylline, 115; 274C, 103; 267C, 104; 273C, 144; 275C, 183; and 238C, 320.

*Sympatholytic Activity.* Compound 267C was the only member of the series exhibiting any antagonism to the pressor action of epinephrine.

*Isolated Rat Uterus.* All of the piperidyls depressed the activity of this preparation to some degree but the effective concentration of the most active compounds, 275C and 238C, was about one hundred times greater than that required to depress isolated rabbit intestine.

*Antihistaminic Action.* Activity as measured upon isolated guinea pig ileum was definite but less than one five-hundredth of that of chlorothen citrate (Tagathen) (N,N-dimethyl-N'-(2-pyridyl)-N'-(5-chloro-2-thenyl)-ethylenediamine citrate) with little variation between members of the series.

*Local Anesthesia.* Local anesthetic action was determined in cats' eyes by the Von Frey hair technique and in the guinea pig skin after intradermal injections. All members of this series except 237C produced some anesthesia in the eye. Compound 238C was the most potent and produced activity of the same order as cocaine. Arranged according to descending activity the order and rating of the compounds were: 238C, 10; 267C, 9; 274C, 6; 273C, 5; 275C, 1; 237C, 0. Some evidence of irritation to the cornea and conjunctiva was observed with all of the compounds. Only 274C and 275C produced anesthesia by intradermal injection in the guinea pig. However, both compounds caused irritation and this factor rendered an assessment of local anesthetic action difficult.

**DISCUSSION.** A comparison of some of the pharmacological properties of this series of compounds has been summarized in table 3. These values were estimated from dose-action relationships; atropine sulfate was given the arbitrary rating of 100.

Correlation of structure and action in this group of piperidyl propanols permits the conclusion that the cyclohexyl substituent is more potent than the propyl, isopropyl, butyl, isoamyl or the phenyl group in enhancing antispasmodic activity, sialoschisis, mydriasis and cardio-vagal inhibition. The cyclohexyl analogue (275C) is consistent in the proportionality between dose and response and in the reliability of the response to a constant dose. Comparison of the activities of atropine sulfate and 275C indicates that as an antispasmodic atropine sulfate is two times as strong as 275C on the isolated intestine and

six times as strong in Thiry-Vella dogs. However, it is eight times as strong as an antisialogogue, three times as strong as a mydriatic and ten times as strong as a cardio-vagal inhibitor. These correlations indicate that the value of 275C as a rival of atropine sulfate will be determined only by a comparison of the actions of effective doses in a given syndrome.

Compound 267C, though a weaker spasmolytic than 275C, is consistent in this effect and has a low incidence of side actions.

Although the tests for chronic toxicity did not reveal undesirable characteristics in any of the compounds the narrow margin between the excitant and

TABLE 3

*Some pharmacodynamic relationships of the piperidyl alcohols, atropine sulfate and trasentin*

The values were calculated from dose-action relationships; atropine sulfate was given the arbitrary value of 100. Doses were administered intravenously to dogs and intraperitoneally to cats.

COMPOUND	SIALO- SCHESIS	CARDIO- VAGAL INHIBITION	ANTISPASMODIC ACTIVITY		MYDRIASIS (INTENSITY)
			Isolated Intestinal segment†	Thiry- Vella†	
	(Dog)	(Dog)	(Rabbit)	(Dog)	(Cat)
Trasentin*.....	0	0	2.4	1.0	0
237C.....	0.7	1.6	11.4	5.0	2.8
267C.....	0.7	3.3	12.5	4.0	2.8
273C.....	1.3	3.3	12.1	5.0	2.1
274C.....	1.3	1.6	18.2	6.0	15.8
238C.....	4.0	5.0	36.4	5.0	15.2
275C.....	13.0	10.0	47.1	16.0	35.0
Atropine sulfate.....	100.0	100.0	100.0	100.0	100.0

\* In the tests for sialoschesis and cardio-vagal inhibition the dose was 0.5 mgm./kgm. and for mydriasis, 2.0 mgm./kgm. Larger doses were not tested.

† Spasm in these preparations was produced by furfuryl trimethylammonium iodide.

antispasmodic doses discourages interest in further study of 237C, 238C, 273C and 274C.

#### SUMMARY

1. The pharmacology of six compounds with the nucleus 3-(N-piperidyl)-1-phenyl-1-propanol with substituents on carbon atom number one has been investigated. The substituents were: cyclohexyl, phenyl, propyl, isopropyl, butyl and isoamyl.

2. The acute intravenous toxicity of these compounds in rats is of the same order of magnitude as that of atropine sulfate. In mice atropine sulfate is approximately one-half as toxic.

3. Extensive studies of chronic toxicity in mice, rats, guinea pigs, rabbits and dogs, did not reveal systemic changes attributable to multiple doses of these compounds.

4. All of the compounds are potent antispasmodics and in varying degrees exhibit other parasympatholytic effects. Like atropine sulfate they stimulate the central nervous system in high doses.

5. The isoamyl derivative, 267C, though the weakest spasmolytic in the group compares favorably with other synthetic antispasmodics. It has a low incidence of side actions and a wide margin between the effective antispasmodic dose and the excitant dose.

6. The strongest antispasmodic in this group is the cyclohexyl derivative, 275C, which approaches atropine sulfate in activity. In the lower doses the parasympatholytic effects are accompanied by a mild depression of the central nervous system and in large doses by excitement. The difference between the depressant dose and the excitant dose is large.

#### REFERENCES

1. BLICKE, F. F.: *J. Lab. & Clin. Med.*, **26**: 131, 1940.
2. CUNNINGHAM, R. W., AND FELLOWS, E. J.: *Fed. Proc.* **1**: 148, 1942.
3. RAYMOND, A. L.: *J. Am. Pharm. Assoc.*, **32**: 249, 1943.
4. BURTNER, R. R.: *Synthetic Antispasmodics*. A. A. A. S. Medicinal Chemistry Section, Gibson Island Conference, 1945. (Separate).
5. WINDER, C. V., KAISER, M. E., ANDERSON, M. M., AND GLASSCO, E. M.: *THIS JOURNAL* **87**: 121, 1946.
6. DENTON, J. J., TURNER, R. J., NEIER, W. B., LAWSON, V. A., AND SCHEDL, H. P.: *J. Am. Chem. Soc.* In press.
7. DENTON, J. J., LAWSON, V. A., NEIER, W. B., AND TURNER, R. J.: *J. Am. Chem. Soc.* In press.
8. DENTON, J. J., NEIER, W. B., AND LAWSON, V. A.: *J. Am. Chem. Soc.* In press.
9. DENTON, J. J., SCHEDL, H. P., NEIER, W. B., AND LAWSON, V. A.: *J. Am. Chem. Soc.* In press.
10. FROMHERZ, K.: *Arch. f. exper. Path. u. Pharmacol.*, **173**: 86, 1933.
11. SCHAUMANN, O.: *Arch. f. exper. Path. u. Pharmacol.*, **196**: 109, 1940.
12. FELLOWS, E. J., AND CUNNINGHAM, R. W.: *Fed. Proc.* **1**: 151, 1942.
13. FOSTER, R. H. K., MOENCH, L. J., AND CLARK, H. C.: *THIS JOURNAL*, **87**: 73, 1946.
14. BECKER, T. J., ANANENKO, E., GLENWOOD, G., AND MILLER, L. C.: *Fed. Proc.* **5**: 163, 1946.
15. BURTNER, R. R., AND BROWN, J. M.: *J. Am. Chem. Soc.*, **69**: 630, 1947.
16. LITCHFIELD, J. T., JR., AND FERTIG, J. W.: *Bull. Johns Hopkins Hosp.*, **69**: 276, 1941.
17. WILCOXON, F., AND MCCALLAN, S. E. A.: *Contrib. Boyce Thompson Inst.*, **10**: 329, 1939.
18. JOHNSON, J. R., AND REYNOLDS, S. R. M.: *THIS JOURNAL*, **59**: 365, 1937.
19. LEE, H. M., SCOTT, C. C., AND CHEN, K. K.: *J. Lab. & Clin. Med.*, **30**: 700, 1945.
20. BURTNER, R. R., AND CUSIC, J. W.: *J. Am. Chem. Soc.*, **65**: 1582, 1943.
21. BURTNER, R. R., AND CUSIC, J. W.: *J. Am. Chem. Soc.*, **65**: 262, 1943.
22. ROBBINS, B. H.: *THIS JOURNAL*, **80**: 264, 1944.
23. FELLOWS, E. J., AND LIVINGSTON, A. E.: *THIS JOURNAL*, **68**: 231, 1940.
24. FELLOWS, E. J., AND LIVINGSTON, A. E.: *THIS JOURNAL*, **74**: 65, 1942.
25. GRUBER, C. M., HART, E. R., AND GRUBER, C. M., JR.: *THIS JOURNAL*, **73**: 319, 1941.
26. LIPSCHITZ, W. L., HADIDIAN, Z., AND KERPCSAR, A.: *THIS JOURNAL*, **79**: 97, 1943.

# THE EFFECTS OF NAPHTHOQUINONES ON GLYCOLYSIS IN MUSCLE EXTRACTS<sup>1, 2</sup>

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Considerable attention has been given to the naphthoquinones in various fields in recent years. In addition to the outstanding discovery that vitamin K has a naphthoquinone nucleus, these compounds have been studied for their antimalarial (1), fungicidal (2), antitubercular (3) and antibacterial actions (4). In addition, some of the naphthoquinones have the power to inhibit mitosis which makes them of interest from the standpoint of tumor growth (5). It has been suggested also that the naphthoquinones may prevent tooth decay by inhibition of acid formation in the saliva (6).

In order to explain these many actions of the naphthoquinones, some work has been done on their possible enzymatic site of action. Potter (7) has shown that 1,4-naphthoquinone and 2-methyl-naphthoquinone inhibit the oxygen uptake of the succinate oxidase system. Ball, Anfinson and Cooper (8) have made an extensive survey of the inhibition by naphthoquinones of the oxygen uptake by the same system and in *Plasmodium knowlesi* and in yeast cells. They came to the conclusion that the inhibitory effect is between cytochrome c and cytochrome b in the chain of respiratory enzymes. Heymann and Fieser (9) used this inhibitor action as a screening procedure for antimalarial compounds but found that the inhibitory action on the succinate oxidase system did not parallel the *in vivo* antimalarial action of some of the naphthoquinones. Wendel (10) has described an inhibition of the oxygen uptake and the use of carbohydrate in erythrocytes parasitized with *P. lophurae*. Fieser and Heymann (11) have extended this work by studying a large number of naphthoquinones for their relative antirespiratory activity. They concluded that the inhibitory action was produced by combination with and deactivation of a respiratory enzyme. Anfinson (12) has demonstrated that the respiration of sea urchin eggs is very sensitive to 2-hydroxy-3(2-methyl-octyl)-1,4-naphthoquinone.

Bueding, Peters and Waite (13) have shown that 2-methyl-1,4-naphthoquin-

<sup>1</sup> Experiments reported before the American Society for Pharmacology and Experimental Therapeutics, Fed. Proc., 7: 220, 1948.

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one inhibits aerobic glycolysis in *Schistosoma Mansonii* *in vitro* and Warren (14) has observed a similar inhibition in bone marrow. Meyerhof and Randall (15) have reported an inhibition of respiration, glycolysis and motility of *Trypanosoma equiperdum* *in vitro*. On purified enzymes, some of the naphthoquinones have been shown to inhibit papain (16) and urease (17).

In view of these many activities of the naphthoquinones, it was decided to test various naphthoquinones on the anaerobic glycolysis of glycogen to lactate in muscle enzyme solutions in order to see if any activity of the naphthoquinones might be on this fundamental process.

**METHODS.** The methods employed were similar to those used by Gemmill (18) in the study of the effects of alloxan on muscle glycolysis. The muscles of the hind legs of frogs were dissected quickly, weighed, and placed in a cooled mortar containing sand. For each gram of muscle 1.5 cc. of cold water were added to the mortar. The mixture was ground and centrifuged. The supernatant fluid was used for the determinations. In each Warburg vessel were placed 1.0 cc. of extract, 0.2 cc. of 2.6 per cent sodium bicarbonate solution, varying amounts of the chemical compound under study and 0.2 cc. of 4.0 per cent glycogen in the side tube. The final volume was adjusted to 2.2 cc. Several vessels were prepared with a similar mixture except that the chemical compound under study was omitted. These mixtures gave the results for normal glycolysis. The vessels were placed in the water bath at 25.8°C. and 95 per cent nitrogen and 5 per cent carbon dioxide were passed through them for six minutes. The gas was discontinued, the side tubes closed and the vessels shaken for another five minutes before the first readings were taken. After these readings, the stopcocks were closed, the glycogen solution was tipped over into the reaction vessel and readings of the carbon dioxide liberated from the bicarbonate solution were made every ten minutes for a period of one hour. The inhibitor was in contact with the enzyme systems for approximately fifteen minutes before the first reading was taken. Results are reported in terms of cu. mm. carbon dioxide liberated per hour. The normal glycolysis was generally of the order of magnitude of 400 cu. mm. of carbon dioxide per hour. Part of this glycolytic activity came from substrates present in the muscle mixture and part from the added glycogen. In fact, the addition of glycogen may be looked upon as a means of saturating the system with substrate for the determinations.

Considerable difficulty was encountered in the use of several of the naphthoquinones on account of their insolubility in water. It was decided to dissolve these compounds in a minimum amount of absolute alcohol, dilute this solution with water and then, if the compound remained in solution in this mixture, to add the alcohol-water mixture to the enzyme in solution. In each case, a control determination was made by adding an equivalent amount of alcohol-water mixture to an enzyme solution and determining the effect of the alcohol on the rate of glycolysis. In these determinations when the final concentration of alcohol exceeded 8 per cent an inhibitor effect on glycolysis was noticed. In any case in which the effect exceeded 10 per cent of the normal value, the experimental results were discarded. No correction was made for the slight change in volume caused by adding water to the alcohol in the calculation of the Molar values. Compounds which were in the form of the hydrochloride were used without additional neutralization. The Molar concentrations are reported in terms of the final concentrations of the compounds in the enzyme solution.

**RESULTS.** The results are presented in tables 1 and 2 and figures 1 and 2. The first naphthoquinone used, sodium 1,2-naphthoquinone-4-sulfonate, proved to be a powerful inhibitor of glycolysis. Numerous experiments were carried out with this compound in order to relate its activity with concentration. The results are given in fig. 1. It can be seen in this figure, that small concentrations of this naphthoquinone increased glycolytic activity slightly, while larger con-



centrations caused a progressive decrease, with practically complete inhibition occurring about  $6 \times 10^{-4}$  Molar solution, although in one experiment complete inhibition was obtained with  $4 \times 10^{-4}$  Molar concentration.

Following these experiments, numerous experiments were made with various naphthoquinones some of which had an inhibitory effect on glycolysis. These results are presented in two tables. Table 1 gives the series of naphthoquinones which gave inhibition while table 2 contains the compounds studied which did not cause inhibition in the concentrations used in these experiments. It is interesting to note in this table that in addition to 2-methyl-1,4-naphthoquinone

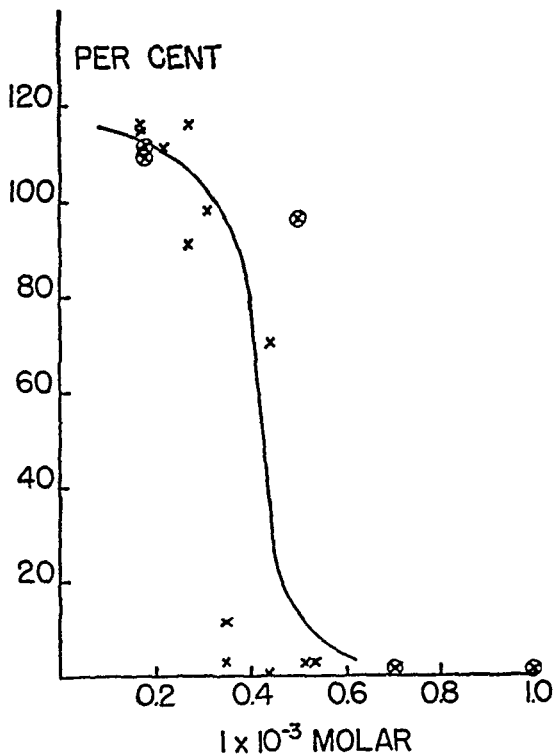


FIG. 1. Effects of increasing concentrations of sodium 1,2-naphthoquinone-4-sulfonate on glycolysis. Abscissae: final concentration of naphthoquinone in enzyme solution; ordinates, per cent of normal glycolysis for one hour determinations; x, single determination; ⊗, average of three determinations.

several other compounds which have vitamin K activity also are inhibitors of glycolysis. These are sodium 2-methyl-1,4-naphthohydroquinone diphosphate and 2-methyl-4-amino-1-naphthol hydrochloride. Another interesting fact is that a halogen attached to the 2 or 3 position increases the inhibitor activity of the molecule. Practically complete inhibition of glycolysis was obtained with  $0.20 \times 10^{-3}$  Molar concentration of 2-methyl-3-bromo-1,4-naphthoquinone in contrast to the use of  $1.6 \times 10^{-3}$  Molar solution of 2-methyl-1,4-naphthoquinone

TABLE 1  
*Naphthoquinones which inhibited glycolysis*

COMPOUND	MOLAR CONC. $1 \times 10^{-3}$	GLYCOLYSIS CU. MM. CO <sub>2</sub> PER HOUR		PER CENT OF NORMAL GLYCOLYSIS
		Without	With	
Sodium 2-methyl-1,4-naphthohydroquinone diphosphate	0.3	474 <sup>2</sup>	420	89
	0.4	474 <sup>2</sup>	408	86
	0.5	474 <sup>2</sup>	383	81
	1.4	373 <sup>2</sup>	264 <sup>2</sup>	71
	2.2	406 <sup>2</sup>	273 <sup>2</sup>	67
2-hydroxy-3-methyl-1,4-naphthoquinone (Phthiocol)	0.6	344 <sup>2</sup>	356 <sup>2</sup>	104
	1.0	383 <sup>2</sup>	202 <sup>2</sup>	53
	1.9	416 <sup>2</sup>	109 <sup>2</sup>	26
2-methyl-4-amino-1-naphthol hydrochloride	0.4	250	321	128
	0.9	250	207	83
	2.0	398 <sup>2</sup>	237 <sup>2</sup>	60
2-hydroxy-1,4-naphthoquinone (Lawsone)	0.3	456 <sup>2</sup>	540	118
	0.5	456 <sup>2</sup>	541	119
	0.6	422	122	29
	0.8	422	388	92
	1.0	422	378	90
	1.6	422	243	58
	1.6	344 <sup>2</sup>	256 <sup>2</sup>	74
1,4 Naphthohydroquinone	0.3	456 <sup>2</sup>	575	126
	0.6	456 <sup>2</sup>	280	61
2-methyl-3-bromo-1,4-naphthoquinone	0.2	330	24	7
	0.3	435 <sup>2</sup>	23	5
	0.03	435 <sup>2</sup>	529	122
	0.003	435 <sup>2</sup>	498	115
	0.5	330	28	9
2-methyl-1,4-naphthoquinone	1.1	392 <sup>2</sup>	146	37
	1.6	349 <sup>2</sup>	20	6
2-chloro-3-N-thiobutyl-1,4-naphthoquinone	0.02	456	526	115
	0.2	456	374	82
	0.3	372	45	12
	0.5	372	17	5
2-methyl-3-thioethyl-1,4-naphthoquinone	0.4	456	394	86
	0.8	456	209	66
2-hydroxy-3-cyclohexanol-1,4-naphthoquinone	0.4	447	358	80
	0.7	447	255	57

Superscripts in table refer to the average of the number of experiments;

in order to obtain the same result. The third fact of interest is that small concentrations produced generally a slight augmentation of glycolysis. Although the response is small (10 to 20 per cent) it occurred in such a large number of experiments that it seems to be a usual event.

Several experiments were made in which cysteine was added to the reaction vessel after the sodium 1,2-naphthoquinone-4-sulfonate inhibition had started. The results of one of these experiments are given in fig. 2. In this experiment, the final concentration of the naphthoquinone was 0.001 Molar, and the final con-

TABLE 2  
*Naphthoquinones which did not inhibit glycolysis*

COMPOUND	MOLAR CONC. $1 \times 10^{-2}$	GLYCOLYSIS CU. MM. CO <sub>2</sub> PER HOUR		PER CENT OF NORMAL GLYCOLYSIS
		Without	With	
1-amino-2-naphthol-4-sulfonic acid	0.2	298 <sup>2</sup>	302	101
	0.3	298 <sup>2</sup>	294	99
	0.4	298 <sup>2</sup>	287	96
	0.5	298 <sup>2</sup>	302	101
2-methyl-3-isothiopropyl-1,4-naphthoquinone	0.4	372	476	128
	0.7	372	451	121
1,4-naphthoquinoyl-2- $\beta$ -thiopropionic acid	0.2	460	580	126
	0.3	460	543	118
	0.5	460	540	117
Lapachol	0.5	330	322	98
Lomatiol	0.3	491	525	107
	0.6	491	450	92
2-methyl-naphthohydroquinone mono-succinate	0.5	364	394	108
	1.0	364	360	99

Superscripts in table refer to the average of the number of experiments.

centration of the cysteine was 0.0017 Molar. The cysteine hydrochloride was dissolved in a sodium hydroxide solution and added ten minutes after the naphthoquinone was placed in the enzyme solution. The experiment was started thirteen minutes later by tipping over the glycogen from the side tubes in the usual manner. It may be seen in the graph that glycolysis was inhibited completely by this naphthoquinone and restored partially by the addition of cysteine.

DISCUSSION. From this study of a number of naphthoquinones and related compounds, it was observed that some of these compounds have the ability to inhibit glycolysis in enzyme solutions. Many questions can be raised from this finding: the site of inhibition, the type of inhibition and the application of these

results to the therapeutic use of the naphthoquinones. Work is proceeding in the laboratory on the site and type of inhibition and will be reported in a later communication.

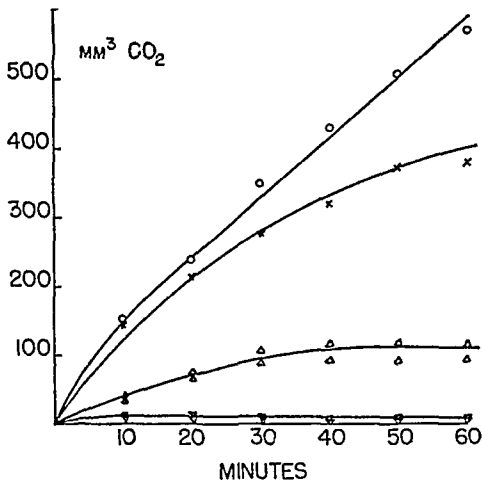


FIG. 2. Effect of cysteine on inhibition of glycolysis produced by sodium 1,2-naphthoquinone-4-sulfonate. Abscissae: minutes; ordinates, cu. mm. of carbon dioxide liberated during glycolysis. Curve (O) glycolysis with cysteine; curve (X), normal glycolysis; curve (Δ), glycolysis with cysteine and naphthoquinone; curve (∇), glycolysis with naphthoquinone.

#### CONCLUSIONS

1. Sodium, 1,2-naphthoquinone-4-sulfonate inhibits glycolysis in muscle enzyme solutions in concentrations of  $0.6 \times 10^{-3}$  Molar. This inhibition is reversed partially by the addition of cysteine.

2. Other naphthoquinones, some of which have vitamin K activity, also possess the property of inhibiting glycolysis in muscle enzyme solutions.

#### REFERENCES

1. FIESER, L. F., AND RICHARDSON, A. P.: *J. Am. Chem. Soc.*, 70: 3156, 1948.
2. Kligman, A. M., AND ROSENWIG, W.: *Invest. Dermat.*, 10: 59, 1948.
3. LLOYD, J. B., AND MIDDLEBROOK, G.: *Am. Rev. Tuberc.*, 49: 539, 1944.
4. COLWELL, C. A., AND McCALL, M.: *Science*, 101: 592, 1945.
5. FRIEDMANN, E., MARRIAN, D. H., AND SIMON-REUSS, I.: *Brit. J. Pharmacol.*, 3: 263, 1948.
6. FOSDICK, L. S., FANCHER, O. E., AND CALANDRA, J. C.: *Science*, 96: 45, 1942.
7. POTTER, V. R., AND DuBOIS, K. P.: *J. Gen. Physiol.*, 26: 391, 1943.
8. BALL, E. G., ANFINSEN, C. B., AND COOPER, O.: *J. Biol. Chem.*, 168: 257, 1947.

9. HEYMANN, H., AND FIESER, L. F.: J. Biol. Chem., 176: 1359, 1948.
10. WENDEL, W. B.: Fed. Proc., 5: 406, 1946.
11. FIESER, L. F., AND HEYMANN, H.: J. Biol. Chem., 176: 1363, 1948.
12. ANFINSEN, C. B.: J. Cell. and Comp. Physiol., 29: 323, 1947.
13. BUEHING, E., PETERS, D., AND WAITE, J. F.: Proc. Soc. Exper. Biol. and Med., 64: 111, 1947.
14. WARREN, C. O.: Am. J. Physiol., 139: 719, 1943.
15. MEYERHOF, O., AND RANDALL, D. O.: Arch. Biochem., 17: 171, 1948.
16. BERNHEIM, F., AND BERNHEIM, M. L. C.: J. Biol. Chem., 134: 457, 1940.
17. GRANT, W. M., AND KINSEY, V. E.: J. Biol. Chem., 165: 485, 1946.
18. GEMMILL, C. L.: Am. J. Physiol., 150: 613, 1947.

# THE EFFECTS OF AMIDINES AND RELATED COMPOUNDS ON GLYCOLYSIS IN MUSCLE EXTRACTS<sup>1, 2</sup>

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Schoenbach and Greenspan, in their recent review on stilbamidine (1), have pointed out that historically the introduction of the diamidine compounds and the study of their chemotherapeutic properties was a direct outgrowth of a search for agents which would block the use of glucose by the trypanosomes. Following the discovery that decamethylenediguandine hydrochloride (Synthalin) was effective against certain trypanosomes, there was a search for less toxic substances. Out of this search came many guanidines, isothiouras, amidines (2) and numerous aromatic diamidines, among them being stilbamidine and pentamidine. It was soon shown, however, that doses of the diamidines which were active against trypanosomes did not produce a fall in blood sugar of the host. Therefore, attention was devoted to the sugar metabolism and oxygen utilization of these organisms. Lourie and Yorke (3) have postulated that the diamidines block the aerobic glucose metabolism in the diamidine-sensitive species. The diamidine-insensitive species, therefore, would be capable of carrying on anaerobic glycolysis in the presence of the drug.

There have been several reports of the effects of these compounds on enzyme systems. Blaschko and Duthie (4) reported an inhibitory action of various amidine derivatives on the amine oxidase activity of the rabbit's liver. Bernheim (5) demonstrated an inhibition by propamidine of the oxidation of proline and alanine by *E. coli*. The oxidation of glucose, pyruvate and succinate is not affected by this drug. Bernheim suggests that the inhibitory effect is not on the cytochrome system. Kopac (6) has stressed the possibility of dissociation of a protamine-nucleate complex with the release of denatured protamine and the formation of an insoluble stilbamidine nucleate as an explanation of the action of stilbamidine. Dickens (7) has demonstrated that guanidine carbonate increases the aerobic glycolysis of the rat brain cortex. This effect was absent or less marked in other tissues. A similar action was obtained with decamethylenediguandine (Synthalin).

Some attention has also been given to the biological effects of the styryl and

<sup>1</sup> A preliminary report of these experiments was made before the American Society for Pharmacology and Experimental Therapeutics, Fed. Proc., 7: 221, 1948.

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cyanine dyes. These compounds have a conjugated system joining the two ring structures together, a structure comparable to that in the molecule of stilbamidine. The biological interest in these compounds dates from 1933 when Browning, Gulbransen, and Niven (8), demonstrated that 2(p-amino styryl)-6-p-acetamido-benzamido-quinoline methoacetate (Styryl 430) produced sarcoma after a single injection of an aqueous solution. Many of the styryl and the related cyanine dyes have antifilarial, anthelmintic and antimalarial activities. There have been several reports of the use of these substances on tissue metabolism and on enzyme systems. Dickens (9) in his study of quinolines on the Pasteur mechanism showed that "Styryl 430" increased respiration and the aerobic and anaerobic glycolysis of brain tissue. Welch *et al.* (10) demonstrated that (1-amy1-2,5-dimethyl-3-pyrrole) (1, 6-dimethyl-2-quinoline) dimethinecyanine chloride decreased the oxygen consumption of *Litomosoides carinii*, a filarial worm. They also noticed an increase in aerobic glycolysis but no effect on anaerobic glycolysis in these worms. Brooker and Sweet (11) tested this same compound on d-amino acid oxidase, the cytochrome system and various dehydrogenases without finding any effect on these enzymes or enzyme systems.

These facts led to the present study of the effects of diamidines and related compounds on anaerobic glycolysis of glycogen to lactate in muscle extracts. Various styryl and cyanine compounds were also tested in this work. This investigation is a continuation of a general study of compounds inhibiting glycolysis: alloxan (12), caffeine and theobromine derivatives (13), and naphthoquinones (14).

The methods used in this study were similar to those used in the previous study with the naphthoquinones (14).

**RESULTS.** The results are given in tables 1 and 2 and in figures 1 to 3. Of the diamidines tested,  $C_{12}$  and  $C_{13}$  inhibited glycolysis while  $C_{11}$  did not slow this process in the concentrations used in these experiments (table 1). Guanidine, methylguanidine, arginine,  $C_8$  and  $C_{13}$  monoguanidines produced inhibition. Of these, the  $C_8$  compound showed marked activity. The diguanidines and diisothiourreas also gave inhibition. Histidine gave no inhibition while azochloramide was an active inhibitor.

In fig. 1 are given the results with stilbamidine and pentamidine. It can be seen in the figure that a minimal concentration of stilbamidine of about  $0.1 \times 10^{-3}$  Molar is necessary for inhibition while increasing the concentration above  $0.3 \times 10^{-3}$  Molar did not reduce the activity of this enzyme preparation below sixty per cent. With pentamidine, the amount of the compound necessary to inhibit glycolysis was greater than with stilbamidine. Chlorguanide (fig. 2) gave a similar result to those obtained with stilbamidine and pentamidine, namely, a definite amount necessary to induce inhibition and incomplete inhibition even with amounts as high as  $1.26 \times 10^{-3}$  Molar. It is also of interest to note that in low concentrations there is a primary inhibition followed by a secondary stimulation which causes the curve of glycolysis with this drug to cross the normal curve after 44 minutes (fig. 3).

With one exception, the styryl and the cyanine compounds (table 2) were

TABLE 1

*Effect of diamidines, mono- and diguanidines, diisothiureas  
and other compounds on glycolysis*

COMPOUNDS	FINAL CONC. $1 \times 10^{-3}$	GLYCOLYSIS CU. MM. CO <sub>2</sub> PER HOUR		PER CENT OF NORMAL GLYCOLYSIS
		Without	With	
Diamidines				
C <sub>11</sub> ·2HCl	0.29	394 <sup>2</sup>	430	109
	0.58	394 <sup>2</sup>	451	114
C <sub>12</sub> ·2HCl	0.28	372 <sup>2</sup>	360	97
	0.56	372 <sup>2</sup>	187	50
C <sub>13</sub> ·2HCl	0.27	372 <sup>2</sup>	320	86
	0.53	372 <sup>2</sup>	260	70
Monoguanidines				
Guanidine HCl	0.95	516 <sup>2</sup>	185	36
	1.90	516 <sup>2</sup>	113	22
	2.86	516 <sup>2</sup>	93	18
	3.81	516 <sup>2</sup>	86	17
Methylguanidine sulfato	0.19	442	456	103
	0.37	442	181	41
	0.19	428 <sup>2</sup>	459	107
	0.37	428 <sup>2</sup>	473	111
	0.56	428 <sup>2</sup>	505	118
	0.75	428 <sup>2</sup>	329	77
Arginine HCl	0.43	420 <sup>2</sup>	109	26
	0.86	420 <sup>2</sup>	70	17
	0.02	362 <sup>2</sup>	314	87
	0.06	362 <sup>2</sup>	366	101
	0.11	362 <sup>2</sup>	354	98
	0.15	362 <sup>2</sup>	358	99
	0.43	433 <sup>2</sup>	148	34
	0.22	433 <sup>2</sup>	157	36
C <sub>8</sub> ·HCl	0.29	348 <sup>2</sup>	20	57
	0.58	348 <sup>2</sup>	5	1
	0.29	348 <sup>2</sup>	118	34
	0.58	348 <sup>2</sup>	125	36
C <sub>11</sub> ·HCl	0.31	430	373	87
	0.62	430	282	66
Diguanidines				
Diguanidine·HCl	0.22*	413 <sup>2</sup>	60	15
	0.44*	413 <sup>2</sup>	92	22
C <sub>12</sub> ·2HCl	0.26	430	256	60
	0.51	430	187	44
Diisothiureas				
C <sub>10</sub> ·HBr	0.20	369	340	92
C <sub>12</sub> ·HBr	0.40	369	17	46
	0.19	369	217	59
	0.38	369	127	34
Other Compounds				
l-Histidine HCl	0.43	420 <sup>2</sup>	440	105
	0.87	420 <sup>2</sup>	418	100
Azochloramide	0.50	428 <sup>2</sup>	18	4
	1.00	428 <sup>2</sup>	42	10



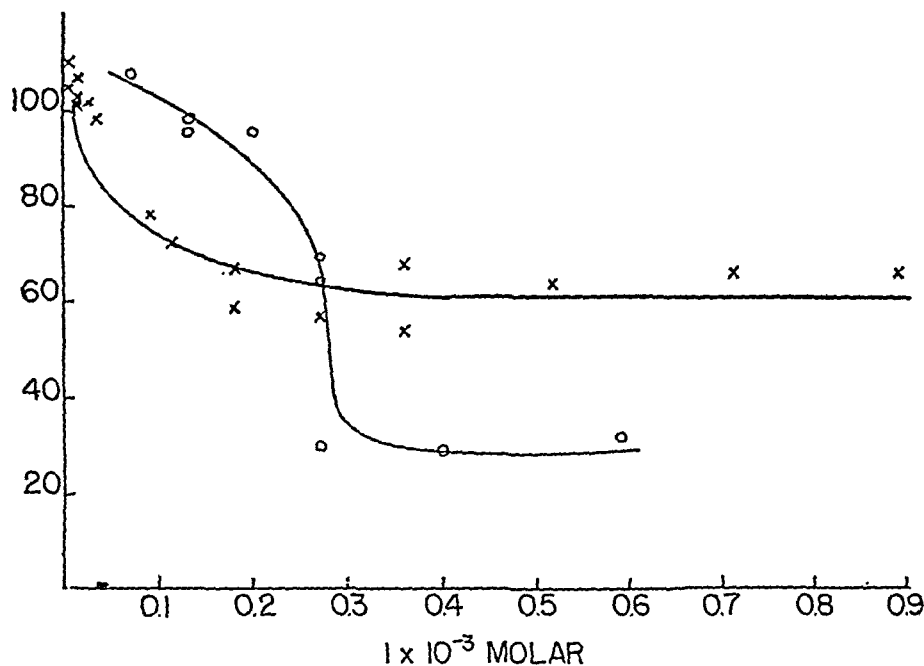


FIG. 1. Effect of stilbamidine and pentamidine on glycolysis. Abscissae, final Molar concentration,  $1 \times 10^{-3}$ ; ordinates, per cent of normal glycolysis. Experiments with stilbamidine (x), with pentamidine (o).

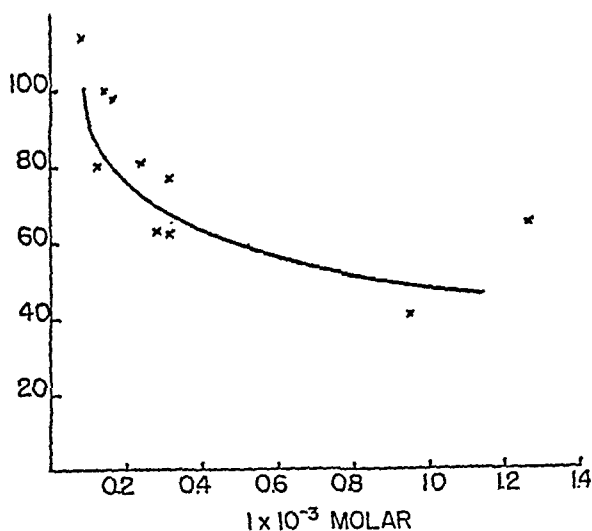
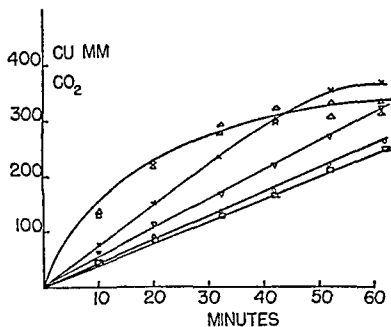


FIG. 2. Effects of chlorguanide on glycolysis. Abscissae, final Molar concentration,  $1 \times 10^{-3}$ ; ordinates, per cent of normal glycolysis.



Fr  
carbo  
(Δ),  
with

minutes, cu mm  
two experiments  
1-2 Molar (O) and

TABLE 2  
*Effects of styryl and cyanine compounds on glycolysis*

COMPOUND	FINAL MOLAR CONC $1 \times 10^{-4}$	GLYCOLYSIS CU MM CO <sub>2</sub> PER HOUR		PER CENT OF NORMAL GLYCOLYSIS
		Without	With	
2(p acetyl amino styryl) 6 dimethylamino quino line methochloride	0 24	428	432	101
	0 48	428	511	119
	0 24	406 <sup>2</sup>	407	100
	0 48	406 <sup>2</sup>	415	102
	0 72	406 <sup>2</sup>	403	99
	0 95	406 <sup>2</sup>	347	85
1 (amyl-2,5 dimethyl 3 pyrrole)(1,6 dimethyl 2 quinoline)dimethinecyanine chloride	0 24	422 <sup>2</sup>	255	60
	0 48	422 <sup>2</sup>	102	24
1' ethyl-3,6 dimethyl 2 phenyl-4 pyrimido 2'- cyanine chloride	0 27	422 <sup>2</sup>	256	61
	0 41	422 <sup>2</sup>	343	81
	0 24	455 <sup>2</sup>	405	89
	0 48	455 <sup>2</sup>	263	59
[2,5 dimethyl 1 phenyl pyrrole (3)](1 methyl pyridine (4))dimethinecyanine chloride	0 28	455 <sup>2</sup>	421	93
	0 56	455 <sup>2</sup>	253	56
1,1' dimethyl 2,2'-carbocyanine chloride	0 25	374 <sup>2</sup>	378	101
	0 50	374 <sup>2</sup>	269	72
4 p diethyl amino styryl pyridine methochloride	0 30	374 <sup>2</sup>	405	108
	0 60	374 <sup>2</sup>	265	71

Superscript 2 refers to the average of 2 experiments

inhibitors to the glycolytic reaction. The inhibitory activity became apparent in concentrations around  $0.5 \times 10^{-3}$  Molar. An attempt was made to use "Styryl 430" in these experiments. When this compound was added to the muscle extract a precipitate formed and the experiments were discontinued.

DISCUSSION. The results demonstrated that certain diamidines, mono- and diguanidines, diisothioureas, styryl and cyanine compounds have the power of inhibiting glycolysis. It is of interest to note that with the majority of these compounds the inhibitory action increased to about fifty per cent of the normal value and then remained constant with increasing concentrations. This fact indicates that the reaction is of a specific nature and not a general denaturation of the proteins in the enzyme systems. Additional work is now being carried out on purified enzyme systems in order to determine the site of the inhibitory action in the glycolytic system.

#### CONCLUSIONS

1. Certain diamidines, monoguanidines, diguanidines and diisothioureas are inhibitors of anaerobic glycolysis in enzyme solutions. Stilbamidine and chlorguanide have this power to a marked degree.
2. Certain styryl and cyanine dyes also inhibit anaerobic glycolysis.

#### REFERENCES

1. SCHOENBACH, E. B., AND GREENSPAN, E. M.: *Medicine*, **27**: 327, 1948.
2. KING, H., LOURIE, E. M., AND YORKE, W.: *Lancet*, **233**: 1360, 1937.
3. LOURIE, E. M., AND YORKE, W.: *Ann. Trop. Med.*, **33**: 305, 1939. (Quoted from Schoenbach and Greenspan, 1)
4. BLASCHKE, H., AND DUTHIE, R.: *Biochem. J.*, **39**: 347, 1945.
5. BERNHEIM, F.: *THIS JOURNAL*, **80**: 199, 1944.
6. KOPAC, M. J.: *Cancer Research*, **6**: 491, 1946.
7. DICKENS, F.: *Biochem. J.*, **33**: 2017, 1939.
8. BROWNING, C. H., GULBRANSEN, R., AND NIVEN, J. S. F.: *J. Path. & Bact.*, **42**: 155, 1936.
9. DICKENS, F.: *Biochem. J.*, **30**: 1233, 1936.
10. WELCH, A. D., PETERS, L., BUEDING, E., VALK, A., AND HIGASHI, A.: *Science*, **105**: 486, 1947.
11. BROOKER, L. G. S., AND SWEET, L. A.: *Science*, **105**: 496, 1947.
12. GEMMILL, C. L.: *Am. J. Physiol.*, **150**: 613, 1947.
13. GEMMILL, C. L.: *THIS JOURNAL*, **91**: 292, 1947.
14. GEMMILL, C. L.: *THIS JOURNAL*, **96**: 166, 1949.

## AMINO ACIDURIA IN URANIUM POISONING

### I. THE USE OF THE AMINO-ACID NITROGEN TO CREATININE RATIO IN "SPOT" SAMPLES OF URINE<sup>1</sup>

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During the course of a comprehensive study of the toxicity of various uranium compounds in this laboratory, it was necessary to develop sensitive diagnostic tests of poisoning. Uranium is a potent nephrotoxic agent which acts on the proximal convoluted tubules, and the tests of poisoning which have been used, such as renal function tests, azotemia, albuminuria, catalasuria and phosphaturia, are all indicative of a disturbed renal function (1). Some of these tests, such as albuminuria and catalasuria are very sensitive and give a positive response following very slight injury; others such as renal function tests and azotemia are indicative of more severe damage. None are specific for uranium poisoning. The azotemia and albuminuria can be determined by relatively simple chemical means, but the renal function tests require special techniques. Catalasuria is detected by a manometric technique and is subject to errors due to contamination unless special care is taken. Because none of these tests was wholly satisfactory by itself, an additional index was desirable which might be useful either by itself or when used in combination with the others.

One of the disturbances associated with uranium poisoning is a marked amino-aciduria (2). Amino acid nitrogen is assiduously conserved by tubular resorption (3), so that only about 2 per cent of that in the glomerular filtrate is normally excreted. A small loss in resorptive power of the proximal tubules should therefore lead to a marked increase in the excretion of amino acids and accordingly amino-aciduria might be a sensitive test of uranium poisoning. Of course, other factors such as food intake and exercise, which also lead to amino-aciduria, must be controlled if amino-aciduria is to be a valid criterion of poisoning. The presence of certain liver or renal lesions and certain metabolic diseases (cystinuria) can also lead to a marked amino-aciduria (4).

The amino acid nitrogen (AAN) concentration of urine can be determined by a simple chemical determination. However, it is the rate of excretion rather than the concentration of AAN which is affected by a tubular poison such as uranium, and the rate of excretion of AAN can be determined only by a simultaneous determination of AAN concentration and of the rate of urine flow. In most studies of excretion rates, a 24-hour urine sample is collected, and the 24-hour

<sup>1</sup> This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

excretion calculated. Shorter periods than 24 hours are often used and would be necessary in this case to avoid post-absorptive urine which is high in amino acid nitrogen. It is also possible to maintain a constant urinary flow by rigidly controlling the water intake. In any case, the measurement of excretion rates is difficult and arduous even in man, where cooperation of the subject can be obtained. In animals, it is more difficult, because of spillage of drinking water, fecal contamination and deterioration and evaporation of cage-collected urines.

In the present paper, a technique is described by which an adequate measure of the rate of AAN excretion can be obtained from random "spot" samples of urine in normal men and in normal, and uranium-poisoned rabbits, avoiding the complication of measuring the rate of water excretion. This end is achieved by using the urinary creatinine concentration as a reference point, and measuring the ratio of AAN to creatinine (AAN/C ratio). Endogenous creatinine excretion is a fairly good measure of glomerular filtration, and uranium, except in lethal doses, has little effect on creatinine excretion as measured by clearance techniques (5). On this basis, the AAN/C ratio in "spot" samples of urine should be a good measure of AAN excretion rates in both normal and uranium-poisoned animals.

**METHODS.** For the human experiments, 5 male laboratory personnel acted as subjects. None had any previous history of renal or hepatic diseases or malfunctions. Overnight urine samples were taken when the men arrived at work, and thereafter, at approximately hourly intervals the bladder was emptied. Records were kept of urinary volumes, amino acid nitrogen and creatinine concentrations. On some days each subject was on a restricted water intake, an *ad libitum* water intake, or a high water intake, so that there was a marked variation in the rate of urine flow. No breakfast was allowed and exercise was restricted to desk work, to avoid amino-aciduria resulting from meals and exercise. At noon the experiments were terminated because it was found that for several hours after the noon meal, the AAN/C ratio was markedly elevated.

In the animal experiments, young (3 to 6 months old) albino rabbits were used. They were fed a diet of Purina Rabbit Chow with water given *ad libitum*. In the few cases where diarrhea developed, oats were added to the diet until recovery was certain. For 24-hour urine collections, the rabbits were kept in regulation metabolism cages fitted with a glass-wool filter at both ends of the urine spout leading into a urine collection bottle containing toluene. The cages were painted with a plastic paint (Ucilon), preventing deterioration of urine which occurs in galvanized metabolism cages. In most of the experiments, spot samples of urine were collected by massage in order to avoid any possibility of contamination or deterioration. Blood samples were obtained for AAN determinations by nicking the marginal ear vein and collecting the blood in oxalated bottles. The marginal ear veins were also used for all injections of uranyl nitrate. The compound was made up in 0.85% saline in the desired concentration and a total volume of about 3 cc. was injected.

The chemical methods used included:

Urinary creatinine—Picric acid method of Folin (6),

Urinary AAN—Method of Albanese and Irby (7),

Plasma AAN—Method of Hoffman (8).

**RESULTS.** In man with rates of urinary flow ranging from 15 to 250 cc./hour, the AAN concentration ranged from 2.5 to 78 mgm. per cent with a relationship which approximates a hyperbola (figure 1). The values of creatinine concentration for the same experiment ranged from 15 to 205 mgm. per cent with a similar

hyperbolic relationship to the rate of urine flow (figure 2). Because the curves for creatinine and AAN are almost parallel, a high correlation must exist between the two variables. In figure 3, in which the AAN and creatinine concentrations for each urine sample are plotted against each other, the correlation is quite apparent. The statistically calculated coefficient is 0.90 with a standard deviation of 0.11, indicating that the result is highly significant. The coefficient of determination is 0.81 (correlation squared) indicating that 81 per cent of the variance in AAN concentration is associated with variance in creatinine concentration and only 19 per cent of the variance is random.

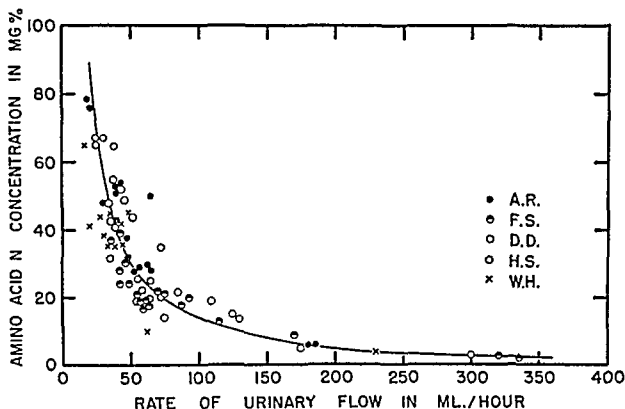


FIG. 1. The effect of the rate of flow of urine on the urinary amino acid nitrogen concentration in male laboratory personnel.

If the ratio of the concentration of AAN and creatinine is taken for each sample of urine (AAN/C ratio), then that fraction of the variance which is common to both should be eliminated. In other words, the AAN/C ratio should show considerably less variance than does either the AAN or creatinine concentration. A rough measure of the variance is given by the range of values from lowest to highest. The AAN and creatinine values vary over a 30 and 15 fold range (figures 1 and 2), whereas the AAN/C values vary over only a  $4\frac{1}{2}$  fold range (figure 4). A better measure of the marked reduction in variance in the ratio is the coefficient of variation (standard deviation divided by mean, times 100). This coefficient is 52.5 and 43.8 for AAN and creatinine respectively, but only 24.5 for the ratio.

The marked reduction in the variance of the ratio results largely from the elimination of variations due to changes in the rate of urinary flow. Although both the AAN and creatinine concentrations are markedly dependent on this factor (figures 1 and 2), the ratio is not nearly so dependent (figure 4). The rates

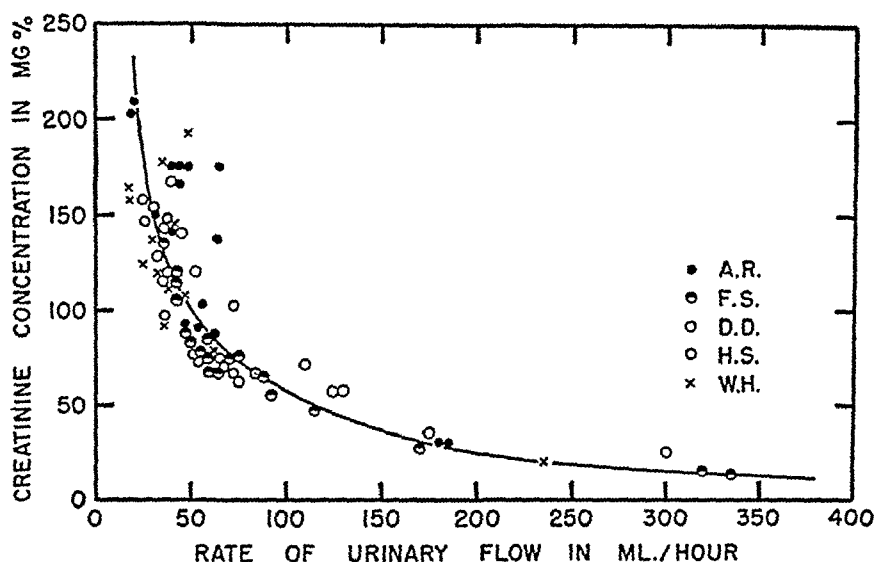


FIG. 2. The effect of the rate of flow of urine on the urinary creatinine concentration in male laboratory personnel.

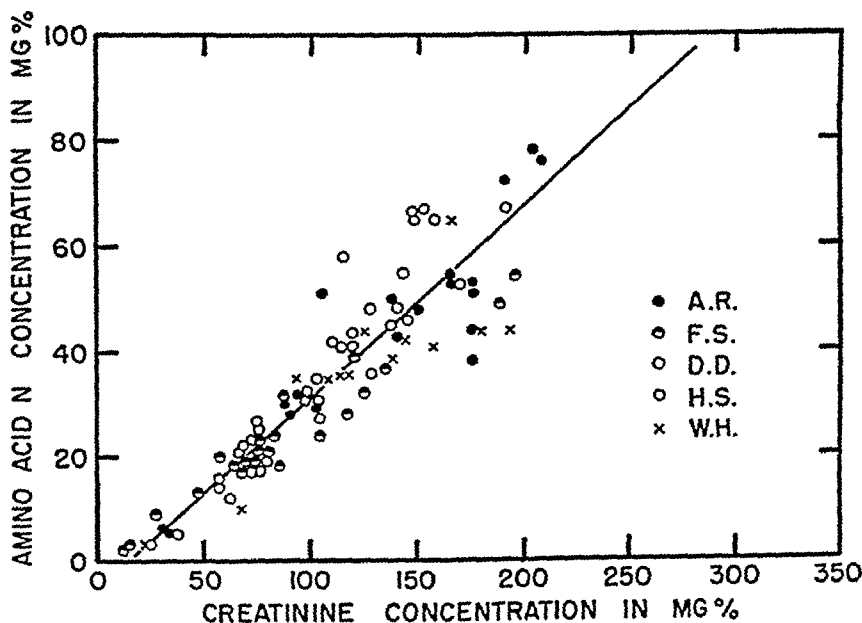


FIG. 3. The relationship between AAN and creatinine concentrations in urine of male laboratory personnel.

of excretion of AAN and creatinine are also independent of the rate of urinary flow (figure 5). The AAN/C ratio should be an excellent measure of the rate of AAN excretion but it has of course the distinct advantage that it can be determined on a "spot" sample of urine, and eliminates the necessity of a 24-hour specimen.

In rabbits, the ratio also showed less variance than did either the AAN or creatinine concentrations. Control data were obtained on 8 rabbits over a 2-week period from daily collections of urine by bladder massage. As in humans there was a significant although lower correlation between the AAN and creatinine concentrations from the same urine sample. This tendency for the two constituents to fluctuate in the same direction is shown graphically in figure 6.

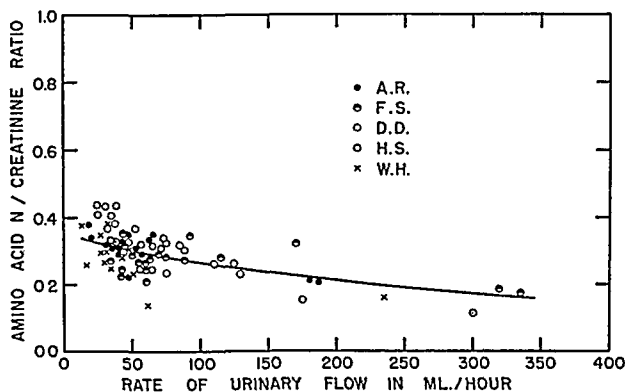


FIG. 4. The effect of the rate of flow of urine on the AAN/C ratio in male laboratory personnel.

The correlation coefficient for the two variables was 0.77, which is highly significant (standard deviation, 0.11). The coefficient of variation was 66 and 58 for AAN and creatinine concentrations, but only 46 for the ratio, a marked reduction. In rabbits, however, the ratio was much more variable than in humans, where the coefficient of variation was only 24.5. The larger variance in rabbits is probably related to the fact that the food intake was *ad libitum* for the rabbit but was rigidly controlled in the case of the humans. The post-absorptive state is known to be associated with higher rates of AAN excretion (4), while the rate of creatinine remains constant.

It seems most probable that in the rabbit, as in the human, the high AAN to creatinine correlation rests on the fact that much of the variation in concentration of these constituents is normally induced by changes in the urinary flow, and



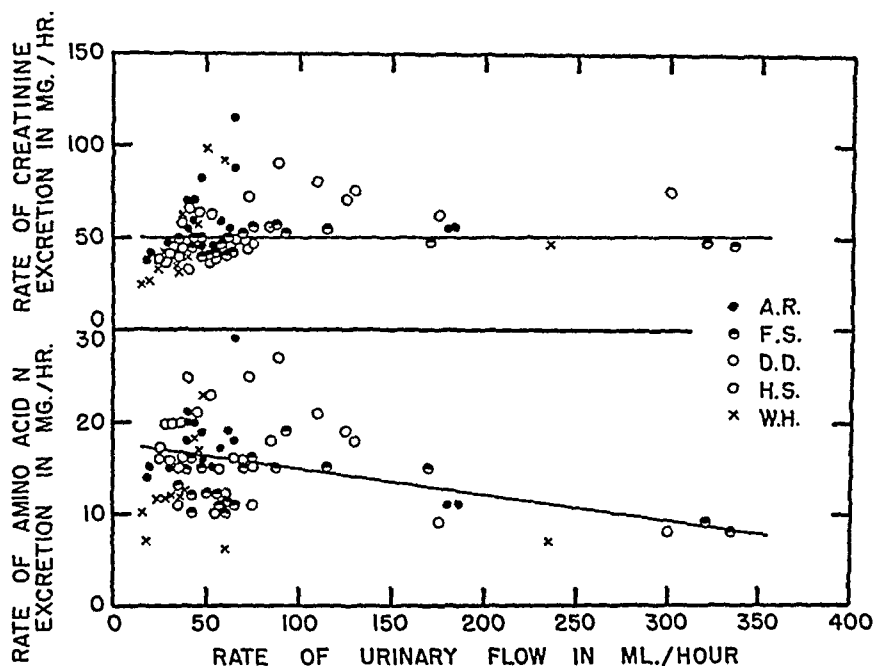


FIG. 5. The effect of the rate of flow of urine on the rate of excretion of AAN and creatinine by male laboratory personnel.

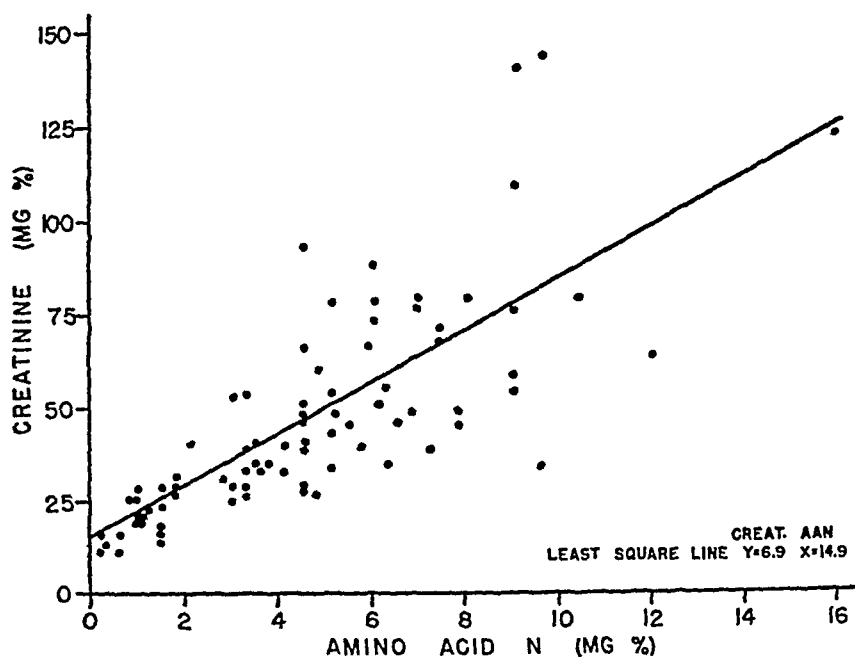


FIG. 6. The relationship between urinary amino acid nitrogen and creatinine concentrations in normal rabbits.

that the ratio is independent of the rate of urinary flow. The ratio eliminates about 59 per cent of the variance as indicated by a coefficient of determination of 0.59 (correlation squared).

*Amino-Aciduria Following U-Poisoning.* It has been shown that U-poisoning is accompanied by an amino-aciduria (2). This was confirmed by a preliminary experiment with 10 rabbits shown in figure 7. Individual values and the mean values of AAN concentration in the urine are plotted for 10 control days and for 5 days following the intraperitoneal injection of 0.3 mgm. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  per kgm. of body weight. Although both the control and post-injection data show considerable variation, the mean values were definitely elevated as a consequence

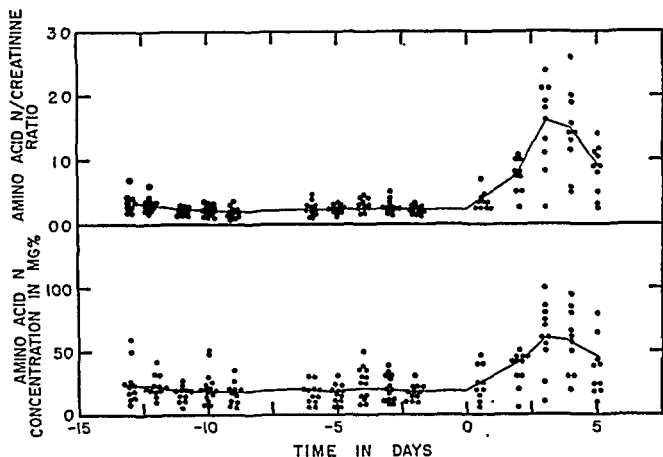


FIG. 7. The effect of a single intraperitoneal injection of 0.3 mgm. of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  per kgm. of body weight on the AAN concentration and AAN/C ratio in urine of rabbits.

of the U-injection. When the same data were plotted as AAN/C ratios, there was a marked reduction in the scatter and a much more striking increase following the U-injection. This can be illustrated numerically in the following manner. In the case of the AAN concentration, 14 of a total of 50 determinations made during the post-injection period were higher than the highest value found during the control period. However, for the AAN/C ratio, 31 of 50 post-injection determinations exceeded the highest control value, more than twice as many. On the basis of the data presented in figure 7, it can be stated that the AAN/C ratio is a more sensitive test for uranium poisoning than is the AAN concentration.

The amino-aciduria associated with U-poisoning seemed to be renal in origin because there was no change in the concentration of AAN in the blood following

administration of U, even though the urinary concentration became abnormally high. Table 1 indicates that rabbits injected intravenously with doses of U as  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  varying from 0.06 to 0.3 mgm./kgm. of body weight showed a 2 to 3-fold increase in the mean urinary amino acid nitrogen concentration, a 5 to 7-fold increase in the AAN/creatinine ratio, but no change in the blood amino acid nitrogen level. During the course of severe and even lethal uranium poisoning in other rabbits, there were no changes in the blood amino acid nitrogen. Contributory evidence indicating that the amino-aciduria is renal in origin was found in preliminary experiments on the tolerance of U-poisoned animals to intravenously injected amino acids. No major alterations were observed in the tolerance curves of glutamic acid and alanine in poisoned rabbits, which seem-

TABLE 1

*The mean amino acid nitrogen levels in urine and blood of rabbits before and after intravenous injections of toxic doses of  $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$*

DAY	NUMBER OF ANIMALS	URINE		BLOOD AAN CONC.
		AAN Conc.	Ratio of AAN to Creatinine	
		mgm. %		mgm. %
-14	8	35.6	—	5.6
-10	8	17.7	0.20	6.4
-3	8	25.4	0.29	6.6
0		Uranium Injected		
+3	8	68.4	1.65	5.8

ingly could handle amino acids in the blood in a normal manner. By contrast, in the amino aciduria due to extensive liver damage, there is failure of the mechanisms of AAN utilization which leads to an elevated blood level of AAN and consequently to an amino-aciduria (4).

#### SUMMARY AND CONCLUSIONS

1. The concentration of AAN in "spot" samples of urine of normal rabbits and humans varied considerably although the rate of excretion of AAN was relatively constant. Most of the variation in the AAN concentration resulted from variations in the rate of flow of urine, and this could be largely eliminated by using the ratio of AAN to creatinine. The AAN/C ratio was as constant as was the rate of excretion of AAN, but required only a "spot" sample of urine rather than a 24-hour sample.

2. The AAN/C ratio was a far better test of uranium poisoning in rabbits than was the AAN concentration.

3. The amino-aciduria resulting from uranium poisoning seems to be renal in origin.

ACKNOWLEDGEMENTS. We wish to acknowledge the cooperation of the 5 subjects who helped in this work. In addition our thanks to Dr. F. Smith who

performed a number of amino acid and creatinine determinations, and to Miss Helen Baldwin who made the statistical analyses.

#### REFERENCES

1. VOEGTLIN, C., HODGE, H. C., ET AL.: N.N.E.S., Div. VI, Vol. IA-Pharmacology and Toxicology of Uranium Compounds, Chapt. XVII.: McGraw Hill, New York, 1949.
2. DEVEZE, R.: *Comp. Rend. Soc. Biol.*, 117: 1111, 1934.
3. PITTS, R. F.: *Arch. Path.*, 140: 156, 1940.
4. PETERS, J. P., VAN SLYKE, D. D.: *Quantitative Clinical Chemistry*, Vol. I.: The Williams and Wilkins Co., Baltimore, Md., 1946.
5. WILLS, J. H.: N.N.E.S., Div. VI, Vol. IA-Pharmacology and Toxicology of Uranium Compounds, Part I, Chapt. V.: McGraw Hill Co., New York, 1949.
6. FOLIN, O.: *Lab. Manual of Biol. Chem.*, 5th Ed., 1934.
7. ALBANESE, A. A., IRBY, I.: *J. Biol. Chem.*, 153: 583, 1944.
8. HOFFMAN, W.: *Am. J. Clin. Path.*, 15: 57, 1945.

# THE EFFECT OF A NITROGEN MUSTARD ON CERTAIN SYNTHETIC REACTIONS *IN VITRO*<sup>1</sup>

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Hutchens *et al.* (1) studied the action of *tris*-(beta-chloroethyl)-amine hydrochloride on *Chilomonas paramecium* and observed that cell division was inhibited before oxygen consumption was depressed. Evidence for the inhibition by methyl-*bis*-(beta-chloroethyl)-amine hydrochloride (MBA) of desoxyribonucleic acid synthesis in amphibian embryos was obtained by Bodenstein and Kondritzer (2). Barron *et al.* (3) reported that both compounds block reactions leading to synthesis. It was of interest therefore to study the action of a nitrogen mustard<sup>2</sup> on relatively simple synthetic reactions which have been shown to proceed in rat liver slices. Those studied were the conjugation of morphine and glucuronic acid (4), of phenol and sulfuric acid (5), of *p*-aminobenzoic acid (PAB) and glycine (6), and urea synthesis. The results show that MBA inhibits most of these reactions, but to different degrees which indicates that separate mechanisms may be involved for each.

**METHODS.** Rat liver was sliced in the usual way and placed in Krebs-Ringer solution (7) saturated with a 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub> mixture, and the reaction was carried out in this atmosphere with the slices suspended in 4 cc. of fluid in 50 cc. Erlenmeyer flasks. Final concentration of MBA varied from  $5 \times 10^{-5}$  *M* to  $5 \times 10^{-3}$  *M*. It was dissolved in Krebs's solution and immediately added to the slices.

**Conjugation of morphine:** The method followed was essentially that used by Bernheim and Bernheim (4). After precipitation of the proteins with 1 cc. 20 per cent trichloroacetic acid, 2 cc. distilled water were added, and the proteins centrifuged. A 1 cc. aliquot was diluted with 9 cc. distilled water. The amount of free morphine remaining was determined colorimetrically according to the method of Snell and Snell (8). A second 1 cc. aliquot was autoclaved 30 minutes at 20 pounds in the presence of 0.1 cc. concentrated HCl. The acid was neutralized with concentrated NaOH, the volume brought to 10 cc. with distilled water, and the amount of morphine, representing both the free and conjugated, was determined.

**Conjugation of phenol:** The procedure reported by Bernheim and Bernheim (5) was employed here. Proteins were precipitated with 1 cc. 20 per cent trichloroacetic acid. Five cc. distilled water were added, and the fluid decanted and centrifuged. A 1 cc. aliquot was added to 8.8 cc. distilled water and 0.2 cc. saturated NaCl solution. The amount of free phenol remaining was determined colorimetrically according to the method of Theiss and Benedict (9). A second 1 cc. aliquot was hydrolyzed 10 minutes in a boiling water bath with 0.1 cc. concentrated HCl. The acid was neutralized with concentrated NaOH, and the volume brought to 10 cc. with distilled water, and the amount of phenol, both free and conjugated, was determined.

**Conjugation of *p*-aminobenzoic acid and glycine:** The procedure used was that reported

<sup>1</sup> This work was aided by a grant from the Duke University Research Council.

<sup>2</sup> The nitrogen mustard was supplied through the courtesy of the Army Chemical Center, Maryland.

by Cohen and McGilvery (6) except that for the extraction of PAB from p aminohippuric acid (PAH) the solutions were shaken in a Kahn shaker (280 oscillations per minute) The color was estimated by the method of Bratton and Marshall (10) The amount of PAH synthesized was calculated according to (6)

Urea synthesis Urea nitrogen was estimated colorimetrically according to the method of Barker (11)

RESULTS Under the conditions of these experiments MBA inhibits most of the reactions studied Both the conjugation of morphine and of phenol were inhibited to about the same extent as seen in table 1 If the concentration of MBA is raised to a sufficient level the morphine conjugation is completely inhibited The synthesis of PAH from PAB and glycine, however, was less affected and even in the presence of a high concentration of MBA the inhibition was not complete Still a different picture was presented in the case of urea synthesis There the degree of inhibition seemed to be related to the source of nitrogen Table 2 shows that urea synthesis from  $(\text{NH}_4)\text{-SO}_4$  is not in-

TABLE 1

*The effect of various concentrations of MBA on the conjugation of morphine, of phenol, and of PAB by rat liver slices*

Data represent the averages of all experiments Figures in parentheses show total number of individual experiments

ADDITION	MICROGM CONJUGATED PER MGM TISSUE (WET WEIGHT)		
	1 mgm Morphine	0.2 mgm Phenol	1 mgm PAB
Control	2.03 (32)	0.265 (15)	5.42 (21)
$5 \times 10^{-4}M$ MBA	1.4 (4)	—	—
$5 \times 10^{-4}M$ MBA	0.92 (27)	0.123 (14)	4.4 (16)
$5 \times 10^{-3}M$ MBA	0.095 (6)	—	1.97 (7)

hibited but when glutamine is used as the nitrogen source inhibition occurs In both cases, however, the increased urea production obtained by the addition of ornithine was completely inhibited by MBA The significance of this is not clear

It was of interest to determine what substances might increase the amount of conjugation, and in turn possibly protect the reactions against the inhibition by MBA The conjugation of morphine was studied in most detail Since it is probable that glucuronic acid combines with morphine, it was assumed that its addition to the reaction might increase the amount of morphine conjugated and/or protect against the inhibition by MBA The results of these experiments with glucuronic acid, pyruvate, lactate, and proteins are shown in table 3 These compounds might have increased the amount of morphine conjugated by serving either as a source of energy (ATP) or as precursors for the glucuronic acid with which morphine combines (lactate and pyruvate) Choline chloride is known to antagonize some of the effects of MBA (3) and thus it might have protected the reaction Desoxyribonucleic acid was studied

as a possible protective agent for the conjugation on the assumption that MBA might combine with it preferentially. In no instance did any of these substances increase the amount of morphine conjugated, and some actually inhibited the reaction. Only protein, whether rat serum, crystalline serum albumin, or casein, protected the reaction against MBA. This might be expected because of the high reactivity of the ethylenimmonium ion with proteins (12).

It was of importance to know if respiration of the slices and conjugation of morphine by the same slices would show a similar degree of sensitivity to any one concentration of MBA. Oxygen consumption was measured for 150 min-

TABLE 2

*The effect of  $5 \times 10^{-4}M$  MBA on urea synthesis by rat liver slices, in relation to the nitrogen source*

Data represent averages of all experiments. Figures in parentheses indicate total number of individual experiments.

ADDITION	MICROGM. UREA SYNTHESIZED PER MCM. TISSUE (WET WEIGHT)	
	4 mgm. $(NH_4)_2SO_4$	8 mgm. Glutamine
Control.....	1.03 (10)	1.36 (10)
$5 \times 10^{-4}M$ MBA.....	1.07 (11)	1.02 (10)
2 mgm. <i>dl</i> -ornithine.....	2.27 (10)	2.13 (9)
MBA plus ornithine.....	1.04 (11)	0.93 (9)

utes of the three-hour incubation period. The 21 parts of 1.3 per cent  $NaHCO_3$  were replaced in the Krebs-Ringer solution by 1.78 per cent  $Na_2HPO_4 \cdot 2H_2O$ . Table 4 shows the averages of two such experiments, each being carried out in duplicate. Values for duplicates checked within ten per cent. It does not seem that respiration is markedly depressed by  $5 \times 10^{-4}M$  MBA. In fact the greatest differences are seen at the 150-minute interval, and even at that point the slices in the presence of MBA show 90.6 per cent of the oxygen consumption of the tissue blank vessels, and 86.3 per cent of that of the slices in the presence of 1 mgm. of morphine. Considering that in these experiments only 47.6 per cent as much morphine was conjugated in the presence of MBA as in the control, it appears that the conjugation is more sensitive to MBA than is the respiration. These results agree with those of Hutchens *et al.* (1).

DISCUSSION. It seems evident that MBA has little effect on the overall respiration of cells in which it markedly inhibits conjugation reactions. This fact and the inability of ATP to overcome MBA inhibition indicates that the energy-yielding systems are not affected and that the drug must act either by preventing the coupling of energy-yielding with energy-utilizing reactions, or by inhibiting the specific enzymes involved. The fact that the conjugation of morphine and phenol are inhibited by much lower concentrations of MBA than is the conjugation of *p*-aminobenzoic acid with glycine suggests that specific enzymes are inhibited. The short life of MBA in solution and its ability to combine with protein is consistent with this. In the case of phenol which

combines with sulfuric acid the actual enzyme which catalyzes the conjugation must be inhibited. With morphine which combines with glucuronic acid it

TABLE 3

*The effect of the addition of various substances on the conjugation of morphine by rat liver slices in the presence and absence of MBA*

Data represent averages of all experiments. Figures in parentheses show the total number of individual experiments.

ADDITION	MICROGM. MORPHINE CONJUGATED PER MGM. TISSUE (WET WEIGHT)	
	Without MBA	$5 \times 10^{-4}M$ MBA
Control.....	2.03 (32)	—
$5 \times 10^{-4}M$ MBA.....	—	0.92 (27)
1 mgm. glucuronic acid.....	1.4 (13)	0.98 (7)
2 mgm. glucuronic acid.....	1.3 (8)	0.85 (4)
3 mgm. glucuronic acid.....	1.1 (4)	0.21 (4)
2 mgm. ATP at start.....	1.0 (4)	0.1 (4)
2 mgm. ATP after 2 hours.....	1.66 (4)	0.73 (5)
8 mgm. ATP at start.....	0.63 (6)	trace (8)
8 mgm. ATP after 2 hours.....	1.26 (6)	0.66 (8)
15 mgm. choline chloride.....	1.60 (4)	1.36 (4)
2 mgm. desoxyribonucleic acid.....	1.43 (3)	0.90 (3)
30 mgm. desoxyribonucleic acid.....	0.56 (4)	0.15 (4)
1 mgm. Na lactate.....	1.60 (6)	0.56 (5)
1 mgm. Na pyruvate.....	1.76 (6)	0.53 (5)
6.5 mgm. serum protein.....	1.60 (6)	1.72 (7)
6.5 mgm. veal infusion medium.....	—	1.46 (5)
6.5 mgm. proteose peptone #3.....	—	1.53 (5)
6.5 mgm. crystalline serum albumin.....	1.92 (3)	1.69 (4)
6.5 mgm. purified casein.....	2.0 (3)	1.70 (4)

TABLE 4

*A comparison of the sensitivity of the conjugation of morphine and respiration of rat liver slices to  $5 \times 10^{-4}M$  MBA*

Data represent averages of two experiments, each being carried out in duplicate. Values for duplicates checked within ten per cent.

	TOTAL O <sub>2</sub> CONSUMED IN CU.MM. AT TIME INTERVALS PER MGM. TISSUE (WET WEIGHT)					MICROGM. MORPHINE CONJUGATED PER MGM. TISSUE (WET WEIGHT)
	minutes					
	30	60	90	120	150	
Tissue blank.....	0.29	0.66	0.96	1.29	1.59	—
1 mgm. morphine.....	0.32	0.69	1.03	1.36	1.67	1.85
morphine plus 5 × 10 <sup>-4</sup> M MBA.....	0.31	0.64	0.94	1.21	1.44	0.88

is possible that the formation of the latter is interfered with. However, addition of glucuronic acid, or pyruvic and lactic acids, which may be its precursors, does not overcome the MBA inhibition which indicates that the drug has in-



hibited the enzyme responsible for the conjugation. Finally, desoxyribonucleic acid does not protect the cell against the action of MBA which suggests that the drug does not react directly with the nucleic acid part of proteins.

#### SUMMARY

1. Inhibition of the conjugation of morphine and glucuronic acid, of phenol and sulfuric acid, and of *p*-aminobenzoic acid and glycine, and of urea synthesis by methyl-*bis*-(beta-chloroethyl)-amine HCl (MBA) is described.

2. Variations of the sensitivities of these reactions to the same concentration of MBA were found.

3. None of the compounds tested increased the amount of morphine conjugated, nor did any with the exception of protein overcome the MBA inhibition. Protein in the reaction mixture offered partial protection.

4. MBA inhibits the conjugation of morphine in concentrations which have little effect on respiration.

#### REFERENCES

1. HUTCHENS, J. O., PODOLSKY, B., and McMAHON, T. M.: *Fed. Proc.*, **7**: 59, 1948.
2. BODENSTEIN, D., and KONDRITZER, A. A.: *J. Exper. Zool.*, **107**: 109, 1948.
3. BARRON, E. S. G., BARTLETT, G. R., MILLER, Z. B., and SEEGMILLER, J. E.: *J. Exper. Med.*, **87**: 503, 1948.
4. BERNHEIM, F., and BERNHEIM, M. L. C.: *THIS JOURNAL*, **81**: 374, 1944.
5. BERNHEIM, F., and BERNHEIM, M. L. C.: *THIS JOURNAL*, **78**: 394, 1943.
6. COHEN, P. P., and MCGILVER, R. W.: *J. Biol. Chem.*, **166**: 261, 1946.
7. KREBS, H. A., and HENSELEIT, K.: *Z. Physiol. Chem.*, **210**: 33, 1932.
8. SNELL, F. D., and SNELL, C. T.: *Colorimetric Methods of Analysis*, II, p. 510, 1936. Van Nostrand.
9. THEISS, R. C., and BENEDICT, S. R.: *J. Biol. Chem.*, **61**: 67, 1924.
10. BRATTON, A. C., and MARSHALL, E. K.: *J. Biol. Chem.*, **128**: 537, 1939.
11. BARKER, S. B.: *J. Biol. Chem.*, **152**: 453, 1934.
12. GILMAN, A., and PHILIPS, F. S.: *Science*, **103**: 409, 1946.

# EFFECTS OF PHENOBARBITAL ON OXYGEN CONSUMPTION OF BRAIN SLICES

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In the literature two types of investigations have been reported on the mechanism of action of the barbiturates as related to oxygen uptake, namely, anesthetic doses *in vivo*, and relatively high concentrations *in vitro*. In certain *in vivo* experiments, for example, it has been shown that the oxygen uptake of the monkey brain (1) and of dog brain (2) under light anesthesia is greater than the comparable oxygen uptake under deep anesthesia. It is quite possible, however, that the differences between those values and a true normal value (no anesthesia) may have been of very much less magnitude. Using brain slices other workers (3-5), have shown a depression of the  $Q_{O_2}$  by barbiturates. Furman and Field (5) working with graded concentrations of barbiturates, showed a quantitative relationship between the concentration of some of the barbiturates necessary to produce a 50 per cent depression of the  $Q_{O_2}$  in rat brain slices and a concentration sufficient for deep anesthesia in the rat. This comparison is based on the assumption that the rat brain tissue contains four times as much barbiturate as the other soft tissues of the rat during barbiturate anesthesia, as shown by the work of Tatum, Nelson and Kozelka (6). However, as practically all of the barbiturates (with the exception of the thiobarbiturates) are used almost exclusively as sedatives or hypnotics, it seemed that more detailed studies of the action of very much lower concentrations of barbiturates, which presumably would produce less depression, on the oxygen consumption of brain slices might yield valuable information on the possible mechanism of action of this group of drugs. A recent comparison of the depression of the oxygen uptake of tissue slices of cerebral cortex with that of the thalamus (rats) in the presence of relatively low concentrations of pentobarbital sodium, revealed that the responses often were quite variable, if the slices were subjected to concentrations of pentobarbital near the minimum necessary to produce a depression of the  $Q_{O_2}$  (7).

Accordingly, experiments were planned to study the effects of certain low concentrations of phenobarbital sodium on the  $Q_{O_2}$  of rat brain slices.

**METHOD.** The direct method of Warburg (Umbreit, Burris and Stauffer, 8) was used in which the  $Q_{O_2}$  of rat cerebral cortex slices respiring in Krebs-Ringer-phosphate solution carrying 100 mgm. per 100 cc. glucose was measured at 37°C. Specifically, the rat was decapitated, the brain removed, and placed (not to exceed two minutes after decapitation) in a small, moist, covered glass container submerged in finely chopped ice, where the tissue chilled for about three minutes. Then, the brain was removed from the container, placed on an ice cold glass surface, and sufficient slices (thickness, approximately 0.4 mm.) were cut quickly, using a template method, to total about 25 mgm. of tissue. The brain was

replaced immediately in the cold chamber, and the cut slices which were placed (as cut) on the dry surface of an inverted small glass weigh-bottle top were weighed in the weigh-bottle. After the few seconds required for the slicing and weighing, the slices were transferred quickly to the medium in the flask which had been resting in finely chopped ice. Each flask contained exactly 2.0 cc. of Krebs-Ringer-phosphate-glucose solution (pH 7.2, glass electrode). The center well contained 0.2 cc. of 10 per cent KOH, with filter paper wick to increase the efficiency of the  $\text{CO}_2$  absorption. The flasks were gassed with oxygen and allowed to equilibrate 15 minutes (shaking rate 115 per minute) before the first reading was taken. An aliquot sample was weighed, dried at  $105^\circ\text{C}$ . and weighed again to determine the wet/dry weight ratio. This procedure was repeated for each flask of the series. Not more than five flasks were supplied with tissue from one brain.

To check the methods, solutions, slicing technique, etc., the  $\text{QO}_2$  values of 20 individual rat cortices were determined during the first 30 minutes and compared with the values obtained during the succeeding 30 minutes.

The effects of graded concentrations of phenobarbital sodium on the  $\text{QO}_2$  of a second group of rat cortices were determined by using tissue in one flask as a control respiring in the Krebs-Ringer-phosphate-glucose solution, and another flask of tissue (from the same cortex) respiring in the same solution plus the phenobarbital. This method is valid, for Read (7) and the writer have observed that the  $\text{QO}_2$  is steady for more than one hour under the conditions outlined above while the slices are under the influence of a barbiturate. Concentrations of 1, 5, 10, 20, 30, 40 and 50 mgm. per 100 cc. phenobarbital sodium were tested in this part of the study reported here. The cortices of ten rats were used for each of the 20, 30, 40 and 50 mgm. per cent concentrations, and 20 animals were used for each of the 1, 5 and 10 mgm. per cent concentrations.

As the 1 mgm. per cent concentration seemed to increase the rate of oxygen consumption rather than depress it, a third group of rats was used in a slightly different experiment. In this series, the  $\text{QO}_2$  values were determined for the first 30-minute period in Krebs-Ringer-phosphate-glucose solution alone. Then 0.2 cc. of the latter solution carrying the proper amount of phenobarbital sodium was tipped in from the side arm of the Warburg flask and the  $\text{QO}_2$  measured during the next 30 minutes. Graded concentrations of the barbiturate (0.1, 0.2, 0.3, 0.5, 1.0 and 5.0 mgm. per cent) were studied by means of this "tip-in" technique. Samples from ten different rat cortices were used in each of the concentrations of 1 and 5 mgm. per cent and 20 to 30 cortices were represented at each of the lower concentrations (0.1, 0.2, 0.3, and 0.5 mgm. per cent).

**RESULTS.** 1. Control experiments. Comparison of the average (20 different rat cortices)  $\text{QO}_2$  value (11.7) during the first 30 minutes with the  $\text{QO}_2$  value (11.6) during the succeeding 30 minute period reveals practically identical respiratory rates (fig. 1). The straight line graph indicates that the first 30 minutes can be used as a control period for the study of the effects of phenobarbital tipped in (at the end of the 30 minutes), in the second group of experiments.

2. Experiments with phenobarbital sodium. The results are presented in a scatter diagram with the arithmetic mean values (fig. 2) graphed as per cent of the control  $\text{QO}_2$  (considering  $\text{QO}_2$  during the 30-minute control period as 100 per cent) against concentration (in mgm. per cent) of the phenobarbital on a logarithmic scale.

The effects of the concentrations of 20-50 mgm. per cent inclusive (pH of the strongest concentration used did not exceed 7.3) followed the same pattern of depression of the  $\text{QO}_2$  as has been reported in the existing literature (4, 5). However, 10 mgm. per cent caused an increase in the oxygen consumption in

four cases out of twenty, with no change in one case, and a decrease in fifteen cases. Five mgm. per cent inhibited the rate of oxygen consumption in practically half of the experiments but increased it in about half of the cases. The ten additional experiments in which the 5 mgm. per cent of phenobarbital sodium was tipped in after 30 minutes, yielded similar results. Adding these latter to those in the preceding experiment revealed an average of less than one per cent change from the control values on a basis of the 30 cases. Twenty-two of 30 cortices showed an increase in oxygen consumption in the presence

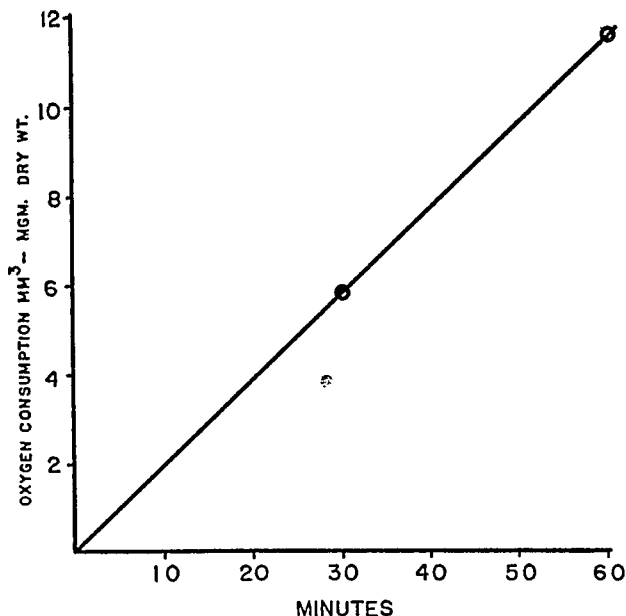


FIG. 1. MEAN OXYGEN CONSUMPTION RATES OF CEREBRAL CORTEX SLICES FROM TWENTY RATS

of one mgm. per cent and eight, a reduced consumption, resulting in a mean  $Q_{O_2}$  increase of four per cent. Twenty cases subjected to each of the concentrations of 0.2, 0.3 and 0.5 mgm. per cent out of the twenty tested at each of those three concentrations (all tip-in experiments) produced an increase in the rate of oxygen uptake, with mean values of 8, 12 and 8 per cent increase, respectively. The mean value based on 30 cases at 0.1 mgm. per cent of the

phenobarbital sodium (all tip-in experiments) was less than one per cent from that of those same tissues during the first 30-minute control period.

**DISCUSSION.** One clear cut finding is observed from the data, that is, low concentrations of phenobarbital sodium (0.2, 0.3 and 0.5 mgm. per cent) definitely increased the oxygen uptake of brain slices in all 60 experiments (20 at each point). This is not too surprising in view of the well known stimulation

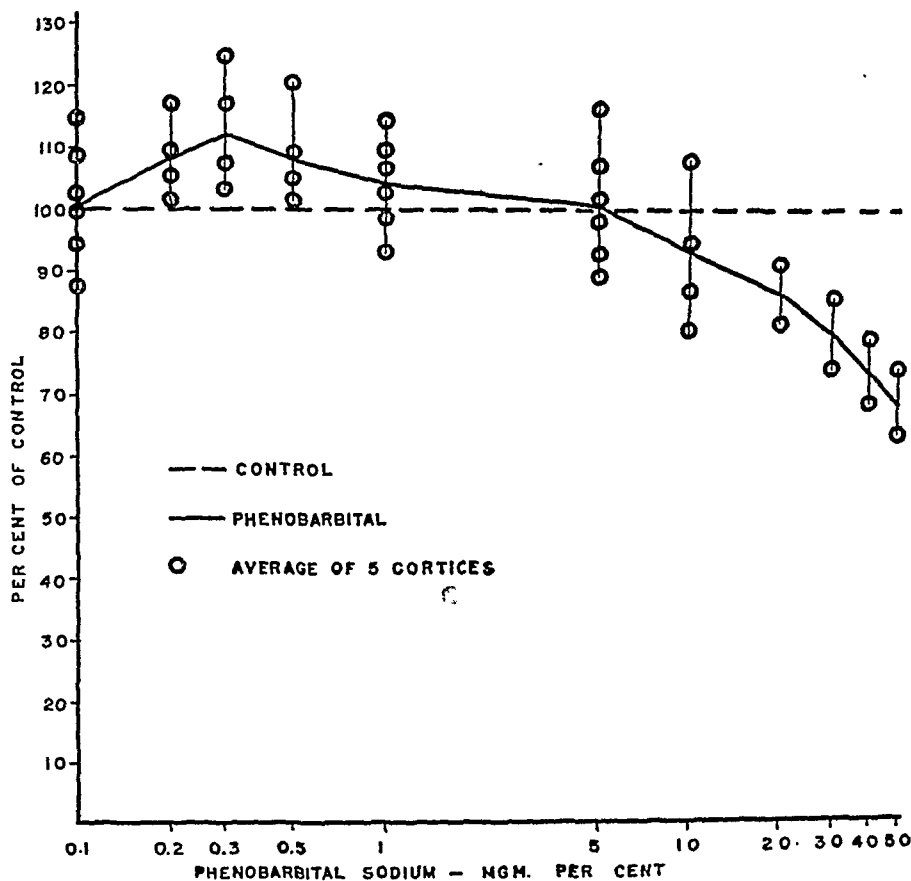


FIG. 2. EFFECTS OF PHENOBARBITAL SODIUM ON OXYGEN CONSUMPTION OF SLICES FROM THE CEREBRAL CORTEX OF THE RAT

of intact animals by small doses of anesthetics. Furthermore, it has been reported recently by Bodine and Fitzgerald (9) that low concentrations of urethane produce a marked increase in the oxygen consumption of grasshopper embryos (*M. differentialis*), while high concentrations reduce the oxygen consumption.

This increase in oxygen consumption by brain slices (induced by barbiturates) is very interesting, because the amount of the phenobarbital sodium given in one dose as a sedative or hypnotic (U.S.P. XIII, dose 30 mgm.) to an average man is so small that, if the drug were distributed equally throughout the soft

tissues, the concentration would be extremely low in the cortex. Even if 100 mgm. should be given, as is done frequently in therapeutics, and if the brain contained four times as much as other tissues (as reported by Tatum, Nelson and Kozelka for rabbits, 6) the dilution by the soft tissues would still be so great that one could hardly expect a depression of the  $Q_{O_2}$  on a basis of the *in vitro* studies.

Therefore, these data do not indicate any relationship between the depression of the  $Q_{O_2}$  in brain slices and mechanism of action of phenobarbital as a sedative or hypnotic *in vivo*. However, this study does not eliminate the possibility that certain centers or other small areas in the brain might be so sensitive that the  $Q_{O_2}$  could be depressed by extremely low concentrations, which concentration might even stimulate the cortex as a whole, or as slices *in vitro*.

The mechanism of the increased oxygen consumption of the brain slices induced by the barbiturate in the present study is not understood.

#### SUMMARY

Concentrations of 0.2, 0.3 and 0.5 mgm. per cent phenobarbital sodium definitely increased the oxygen consumption of slices of the rat cerebral cortex.

#### REFERENCES

1. SCHMIDT, C. F., KETY, S. S., AND FENNES, H. H.: *Am. J. Physiol.*, **143**: 33, 1945.
2. HOMBERGER, E., HIMWICH, W. E., ETSTEN, B., YORK, G., MARCSCA, R., AND HIMWICH, H. E.: *Am. J. Physiol.*, **147**: 343, 1946.
3. SEN, K. C.: *Biochem. J.*, **25**: 849, 1931.
4. QUASTEL, J. H., AND WHEATLEY, A. H. M.: *Proc. Roy. Soc. (London)*, **B112**: 60, 1932.
5. FURMAN, F. A., AND FIELD, J.: *THIS JOURNAL*, **77**: 392, 1943.
6. TATUM, H. J., NELSON, D. E., AND KOZELKA, F. L.: *THIS JOURNAL*, **72**: 213, 1941.
7. READ, W. O.: Unpublished
8. UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F.: *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 203, Burgess Pub. Co., Minneapolis, 1945.
9. BODINE, J. H., AND FITZGERALD, L. R.: *Physiol. Zool.*, **22**: 308, 1948.

# AMINO ACIDURIA IN URANIUM POISONING

## II. THE RESPONSE TO DIFFERENT AMOUNTS OF URANIUM GIVEN INTRAVENOUSLY AND BY INHALATION<sup>1</sup>

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Because of the present interest in the toxicology of uranium, it has been necessary to develop sensitive tests of poisoning which could be applied both in experimental animal work and in industrial human exposures. The prerequisites of a good test are, of course, simplicity, sensitivity and specificity. Because uranium is primarily a nephrotoxic agent (1), poisoning by this agent is accompanied by alterations in certain blood and urine constituents such as blood non-protein and urea nitrogen, and urinary protein catalase, phosphatase and sugar among others (2, 3), all measures of an altered renal function.

Another disturbance which accompanies uranium poisoning is amino aciduria (4). It has been suggested that changes in the rate of amino acid nitrogen (AAN) excretion might be a useful test of uranium poisoning (5). Rates of excretion of AAN are difficult to determine especially in experimental animals. However, it is possible to obtain a measure of the rate of excretion of AAN by using the ratio of AAN to creatinine concentrations (AAN/C ratio) in "spot" samples of urine, avoiding the necessity of collecting a 24-hour urine sample. The AAN/C ratio is as constant as is the rate of excretion of AAN or creatinine, is independent of urine flow, and is markedly increased during uranium poisoning (5).

In the present paper, the AAN/C ratio is evaluated as a test for uranium poisoning. The minimal dose of uranium necessary to give a response is determined. The time course of the response is shown for single and repeated intravenous injections of uranium and for daily inhalation of uranium dusts, and the AAN/C ratio is compared with other tests of poisoning.

**METHODS.** Young (three to six months old) albino rabbits were used in most of the experiments. They were fed a diet of Purina Rabbit Chow with water given *ad libitum*. In the few cases where diarrhea developed, oats were added to the diet until recovery was certain. For 24-hour urine collections, the rabbits were kept in regulation metabolism cages fitted with a glass-wool filter at both ends of the urine spout leading into a urine collection bottle containing toluene. The cages were painted with a plastic paint (Ucilon), to prevent deterioration of the urine which occurs in galvanized metabolism cages. In most of the experiments, spot samples of urine were collected by massage in order to avoid contamination which may occur in cage-collected samples. Blood samples were obtained for AAN and nonprotein nitrogen determinations by nicking the marginal ear vein and col-

<sup>1</sup> This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

lecting the blood in oxalated bottles. The marginal ear veins were also used for all injections of uranyl nitrate. The compound was made up in 0.85 per cent saline in the desired concentration and a total volume of about three cc. was injected. The methods used in the inhalation experiments are described in full elsewhere (6).

The chemical methods used included:

- Urinary protein—sulfosalicylic method (7)
- Urinary creatinine—Folin's picric acid method (8)
- Urinary AAN—method of Albanese and Irby (9)
- Urinary catalase—Warburg technique (2)
- Plasma AAN—method of Hoffman (10)
- Blood NPN—method of Folin and Wu (11)
- Uranium—ferrocyanide method (12)

**RESULTS.** *Minimal Dose of Uranium Necessary to Evoke a Response in AAN/C Ratio.* An attempt was made to determine the minimal dose of uranium that would elicit a response in the rabbit in terms of an increase in urinary AAN/C ratio, by injecting intravenously, graded amounts of uranyl nitrate (0.01, 0.02 and 0.04 mgm. U/kgm.). Eight rabbits were given each of these doses in turn at intervals of about three weeks and urines were collected daily by bladder massage. The values for the AAN/C ratio are given for each rabbit in figure 1. Following the injection of 0.01 mgm. of U/kgm., there was little change in the mean value of the ratio. However, after 0.02 and 0.04 mgm. of U/kgm., there was a definite increase for a few days followed by a return to the control level. In view of the transient nature of the elevated AAN/C ratio and in view of the overlapping range of values in the control and experimental periods, it was necessary to evaluate the data statistically. First, a "cutting point" was arbitrarily selected at a level of 0.19, derived from the mean for the control data (0.10), plus two standard deviation units ( $\sigma = 0.045$ ). All values above the "cutting point" were considered abnormal. In figure 1, the "cutting point" is shown as a dotted line. During the control period, only one value of 84 exceeded the cutting point, compared to four of 46, 22 of 87, and 31 of 81 following each of the three uranium injections. If an individual animal showed two values above the "cutting point" in any given period, then that animal was judged to have responded in a positive manner to the test. On this basis, none of eight animals were positive in the control period, one of eight responded to 0.01 mgm. of U/kgm., four of eight to the 0.02 mgm. of U/kgm. and all eight to 0.04 mgm. U/kgm.

In order to make tests of significance, the data of figure 1 are compiled in table 1 in terms of the mean values of the AAN/C ratio, and the fraction of abnormal values during the control and each injection period. The minimal dose of uranium to elicit a statistically significant increase in the ratio was 0.02 mgm./kgm. when the animals were considered as a group. The percentage of values above the cutting point for the ratio during this experimental period (0.02 mgm./kgm. injected) was 25 per cent as contrasted with less than 2 per cent during the control period. The percentage of high values for the group was proportional to the dose of uranium given, but individual animals varied considerably in the level of dosage at which they showed a significant response. For instance, one rabbit



(#2) showed some very abnormal values at a dose of only 0.01 mgm. U/kgm., others responded to 0.02 mgm. of U/kgm., while some required 0.04 mgm. of U/kgm.

*The AAN/C Ratio Compared with Proteinuria as a Sensitive Test of Uranium Poisoning.* The appearance of protein in the urine has been used extensively as a sensitive test for uranium poisoning. The urine samples used for the determination of AAN/C ratio were also analyzed for protein in most cases. A "cutting point" of 10 mgm. per cent of protein in the urine has been established

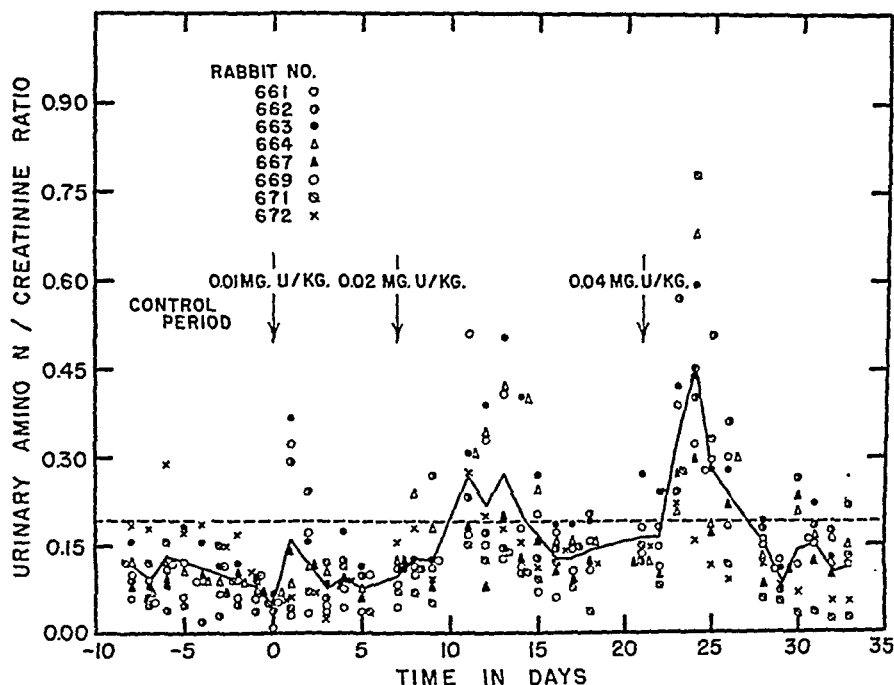


FIG. 1. The effect of various intravenous injections of uranyl nitrate on the AAN/C ratio of eight rabbits.

The heavy black line represents the daily mean. The dotted line is the "cutting point" above which values are considered abnormal. The "cutting point" was arbitrarily selected as the control mean plus two standard deviation units.

by previous work in this laboratory as a level for rabbits, above which an animal can be assumed to have an abnormal proteinuria (3). On this basis, none of a total of 68 determinations were abnormal during the control periods, none of 48 after 0.01 mgm. of U/kgm., one of 87 values after 0.02 mgm. of U/kgm. and eight of 79 after 0.04 mgm. of U/kgm. In terms of the individual animals, none of the eight rabbits showed proteinuria during the control period, none after 0.01 mgm. of U/kgm., one after 0.02 mgm. of U/kgm. and three after 0.04 mgm. of U/kgm. In other experiments, it has been found that 0.1 mgm. of U/kgm. is nearly always followed by proteinuria. Thus the minimal amount of uranium necessary to produce a response is between 0.04 and 0.1 mgm. of U/kgm.

The urinary protein was therefore a less sensitive test of uranium poisoning than was the AAN/C ratio.

*The Course of Amino Aciduria in Rabbits Following Multiple Intravenous Injections of Uranium.* The rabbits which had previously been used in the determination of the minimal dose of uranium necessary to produce amino aciduria were divided into four groups. Four to five repeated injections of uranium nitrate were given at intervals of three to five weeks at levels of 0.04, 0.1 and

TABLE 1

*The AAN/C ratio following intravenous injection of different amounts of uranyl nitrate*

For each animal, the means are given and in addition the ratio of "abnormal" to the total number of determinations (A/T). "Abnormal" values are defined as those more than 2  $\sigma$  (standard deviation) units above the mean for the whole group (above 0.19).

URANYL NITRATE IN- JECTED	RABBIT NUMBER										
	661		662		663		664		667		
	Mean	A/T	Mean	A/T	Mean	A/T	Mean	A/T	Mean	A/T	
mgm U/kgm											
Control	0.07	0/9	0.05	0/10	0.11	0/13	0.09	0/13	0.08	0/10	
0.01	0.05	0/6	0.15	2/6	0.18	1/6	0.10	0/6	0.17	0/5	
0.02	0.23	4/11	0.16	3/11	0.27	7/11	0.24	6/11	0.12	1/11	
0.04.	0.19	4/11	0.25	6/11	0.24	5/9	0.25	3/9	0.17	4/10	
	RABBIT NUMBER										
	669		671		672		All Animals				
	Mean	A/T	Mean	A/T	Mean	A/T	Mean	A/T	P*		
	Control	0.09	0/10	0.11	0/9	0.17	1/10	0.10	1/84	—	
	0.01	0.13	1/6	0.07	0/6	0.08	0/5	0.11	4/46	0.33	
0.02	0.11	0/11	0.10	0/11	0.16	1/10	0.18	22/86	<0.01		
0.04.	0.23	3/10	0.16	3/11	0.12	2/10	0.20	31/81	<0.01		

\* Statistical tests of significance were made between the mean AAN/C ratio during the control period and that for each experimental period, using the "t" test. A P of <0.01 represents a highly significant difference.

0.2 mgm. of U/kgm. body weight for groups 1, 2 and 3. The three to five week period was chosen because histological observations indicate that after a single intravenous injection of uranium, kidney regeneration is complete in about three weeks (13). Analyses of urinary amino acid nitrogen, creatinine and protein were made almost daily during the course of the experiment on spot samples of urine collected by massage. Blood nonprotein nitrogen (NPN) was determined five days after each injection at which time the NPN usually rises to its maximal level following uranium poisoning (2). A fourth group of animals was started at a level of 0.5 mgm. of U/kgm., but these animals all died following the first injection.

The pattern of behavior of the AAN/C ratio was somewhat different for each animal and was dependent on the amount of uranium given. In figures 2, 3 and

4 the data are given for three representative rabbits which illustrate general patterns of response, more or less typical of those seen in other rabbits given the same amounts of uranium.

In the rabbit injected repeatedly with 0.04 mgm. of U/kgm. (figure 2), there was a maximum in the ratio four days after the first injection, followed by a return to lower but still abnormally high values. High values were maintained with no sharp rise after the second injection. After the third injection, however, there was again a maximum on about the sixth day, but after the fourth and

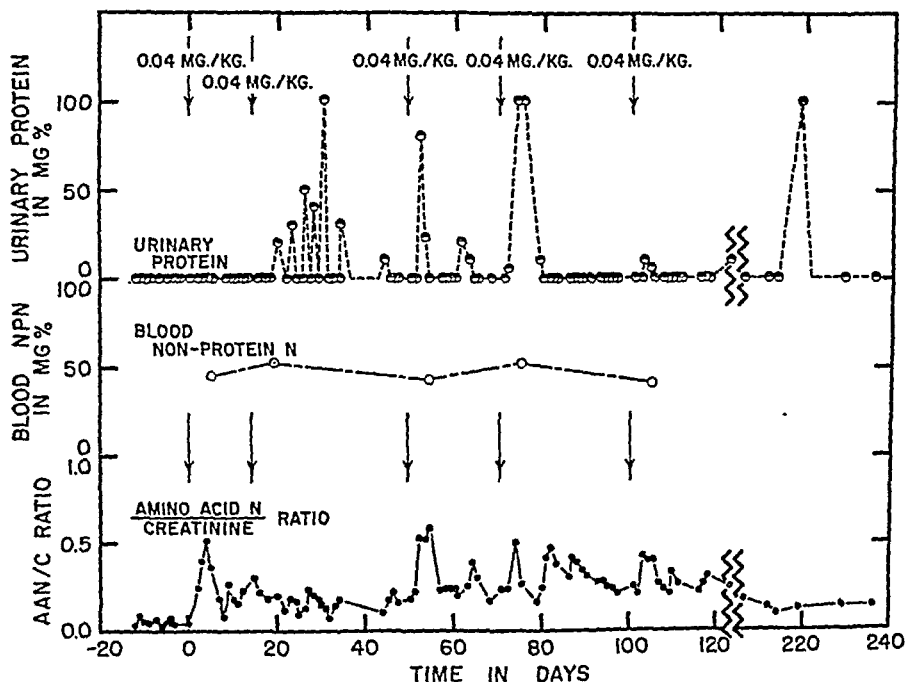


FIG. 2. The effect of repeated intravenous injections of 0.04 mgm. U/kgm. as uranyl nitrate on blood NPN and on urinary protein and AAN/C ratio.

fifth injections there were no further elevations but only a continuing high level through the experimental period.

In the rabbit injected repeatedly with 0.10 mgm. of U/kgm. (figure 3), there was a tremendous peak five days after the first injection followed by a return to lower but still abnormal levels. The second injection also elicited a peak on the fifth and sixth days, but the third and fourth injections did not, although the general level of the ratio was markedly high throughout the remainder of the experiment, higher than in the case of the rabbit injected with 0.04 mgm. of U/kgm.

In the rabbit injected repeatedly with 0.20 mgm. of U/kgm. (figure 4), each injection was followed by a marked peak on the fourth to sixth days, with inter-peak values remaining at very high levels, though tending to fall off gradually.

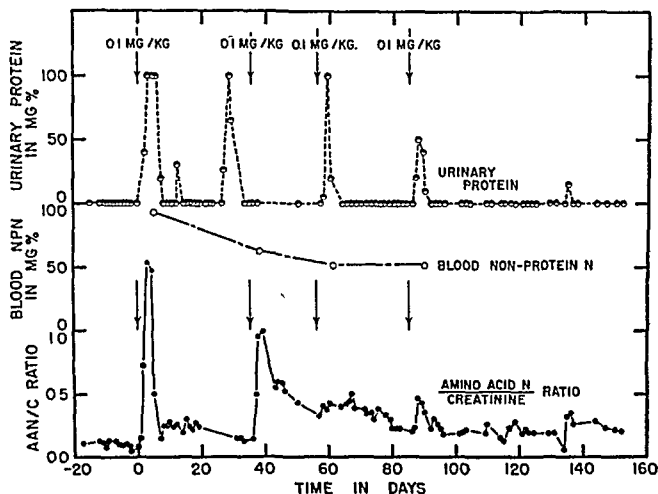


FIG. 3. The effect of repeated intravenous injections of 0.1 mgm. U/kgm. as uranyl nitrate on the blood NPN and on urinary protein and AAN/C ratio.

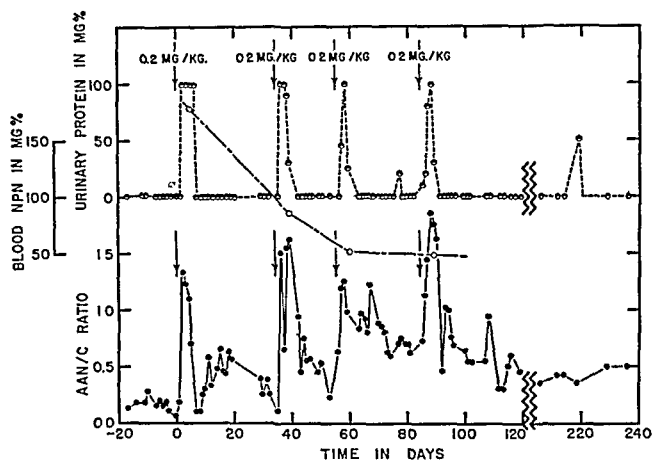


FIG. 4. The effect of repeated intravenous injections of 0.2 mgm. U/kgm. as uranyl nitrate on the blood NPN and on urinary protein and AAN/C ratio.

As pointed out previously, each rabbit studied showed an individual response. Certain generalizations, however, can be made concerning the patterns of behavior. The response to the first injection of uranium at levels between 0.04 and 0.5 mgm./kgm. involves a sharp peak at the fourth to sixth days followed by a return to lower but still abnormally high levels. Even after five weeks the ratio did not return to normal in any animal. In general the height of the peak values and the post-peak values were a function of the amount of uranium injected. With repeated injections there was a definite tendency for the peaks to diminish in size or disappear after the third and fourth injections. This was especially true with the lower doses of U and not so evident with the high doses. However, the inter-peak values in no case returned to normal values during the course of the experiment even two months after the last injection.

Representative animals from each level of injection were sacrificed two months after the last injection for histological examination. Although the ratio of AAN/creatinine was still abnormally high in each animal, no kidney pathology was present at that time. The dosage of uranium used in these experiments was sufficiently high to provoke renal pathology but generally regeneration is complete in about three weeks (13). Thus it must be concluded that the regenerated kidney in the rabbit is less efficient in resorbing amino acid nitrogen than is the normal kidney.

The response of urinary protein to the uranium injections was quite different from that of the AAN/C ratio. In general, each injection of uranium at levels of 0.04, 0.1, 0.2 and 0.5 mgm. of U/kgm. tended to evoke the same response, a proteinuria starting the second or third days after the uranium injection and subsiding after the fifth to ninth days. There were some exceptions to this generalization. After some uranium injections, there was no proteinuria at all, and after others there was sometimes a sustained proteinuria. The extent of the proteinuria was not as well coordinated with the amount of uranium injected as was the AAN/C ratio. The proteinuria tended to be more of an all-or-none response.

There was no increase in the nonprotein nitrogen (NPN) in any of the rabbits following any of the multiple injections at a level of 0.04 mgm. of U/kgm. (figure 2). However, there was an azotemia following injection of 0.1, 0.2 and 0.5 mgm. of U/kgm. In the case of the highest dose (0.5 mgm.), the NPN rose to extremely high values followed by the death of the animals. With 0.1 and 0.2 mgm./kgm. the NPN was elevated following the first injection of uranium but receded with subsequent injections, so that following the second or third repeated injections, there was no elevation at all (figures 3, 4). The decreased response of blood NPN following repeated injections of uranium may well be due to development of tolerance.

*The Excretion of Amino Acid Nitrogen Following Exposure of Rabbits to Inhalation of Uranyl Fluoride Dust.* The appearance of amino aciduria was used as a sensitive test of uranium poisoning during a series of studies of the toxicity of uranyl fluoride ( $\text{UO}_2\text{F}_2$ ) dust by the inhalation route. Animals were exposed six hours per day, six days per week, for five weeks to an atmosphere of the dust

at levels of 2.8, 0.65 and 0.19 mgm. of  $\text{UO}_2\text{F}_2$  per cubic meter of air (6). The concentration of amino acid nitrogen in the urine was determined daily on overnight, cage-collected urine samples of seven to fifteen rabbits in each experiment. The protein and catalase content of the urine was also determined at the same time in some experiments. Blood nonprotein nitrogen was determined semi-weekly.

There was a pronounced amino aciduria in each of the ten rabbits exposed to 2.8 mgm. of  $\text{UO}_2\text{F}_2/\text{m}^3$ . On the average, the amino acid nitrogen concentration<sup>2</sup> began to rise on the fourth day of exposure, continued to rise until the eighth or ninth day and then remained at about four to five times the normal level throughout the exposure period (figure 5). Proteinuria also developed in each of the

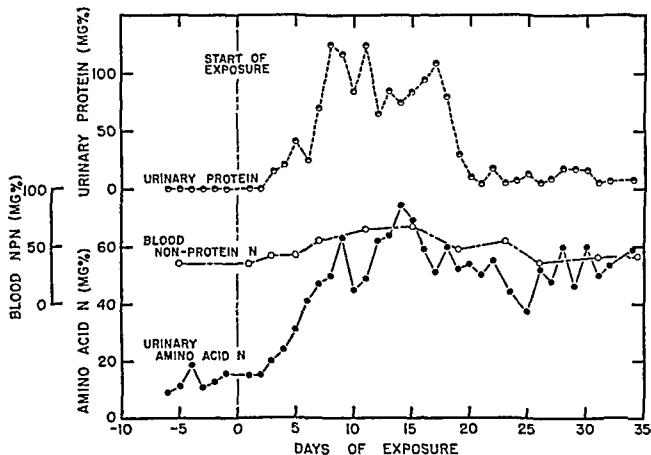


FIG. 5. Changes in blood NPN and in urinary protein and amino acid nitrogen (AAN) during daily exposure to 2.8 mgm. of  $\text{UO}_2\text{F}_2$  dust per cubic meter of air.

ten rabbits starting the third or fourth day, rising to a high level during the seventh to seventeenth days, but then returning to almost normal thereafter, with scattered high values in some of the animals. The blood nonprotein nitrogen showed elevated values in six of the ten rabbits; the mean value increased, starting about the seventh day, and returned to normal about the 21st day.

In the experiment with 0.65 mgm. of  $\text{UO}_2\text{F}_2/\text{m}^3$  of air amino aciduria was not nearly as pronounced. Eight of the ten rabbits showed elevated AAN values. The amino aciduria started about the fifth day and persisted throughout the exposure period at a level about two times that of the control period. A proteinuria was observed in four of the ten rabbits with the maximal response from

<sup>2</sup> The inhalation experiments were done prior to the discovery that the AAN/C ratio of the urine was a better test than the urinary concentration of AAN.

the fourth to fourteenth days, followed by scattered high values thereafter. A catalasuria occurred in six of the ten rabbits, which was somewhat parallel to the proteinuria both in the time course of the response and in the response of individual animals. An elevated blood nonprotein nitrogen was observed only in one rabbit of the ten.

Exposure to 0.19 mgm. of  $\text{UO}_2\text{F}_2/\text{m}^3$  elicited a slight amino aciduria, catalasuria and proteinuria in only one rabbit of fifteen studied.

Certain generalizations can be made on the basis both of the inhalation studies reported here and of many others which are reported in detail elsewhere (14). The most sensitive biochemical tests for uranium poisoning proved to be the urinary AAN and the urinary catalase, with urinary protein somewhat less sensitive and the blood nonprotein nitrogen least sensitive. There was a significant statistical correlation between the appearance of urinary catalase and urinary protein. Both tests tended to be positive in the same animals at the same time. The general time course in each case consisted of a marked peak three to five days after the start of exposure, followed by a return to almost normal with scattered high values throughout the rest of the exposure period. In contrast, the AAN concentration, after an initial response at the fourth or fifth day, remained at an elevated level throughout the whole of the exposure period. The maintained high level is of considerable value in using the urinary AAN as a sensitive test of poisoning, because it allows application of statistical tests of significance to data from the control and exposure periods. In the case of urinary catalase and protein, the elevated values are transient in nature and therefore more difficult to evaluate in borderline cases. There was no statistical correlation between the catalasuria or proteinuria and the amino aciduria. The amino aciduria occurs in different animals at different times and the time course of its response is quite different. Individual animals may show no catalasuria, but a pronounced amino aciduria or *vice versa*. The independence of the amino aciduria from the catalasuria and proteinuria can be used to advantage in testing for uranium toxicity by using all three tests together. For example, in the inhalation experiment with 0.65 mgm. of  $\text{UO}_2\text{F}_2$  per cubic meter of air, eight of ten rabbits showed an amino aciduria, six of ten a catalasuria and four of ten a proteinuria, but all ten animals showed positive to at least one of the tests. Thus all three tests together are more sensitive than any one alone.

Elevation of the blood nonprotein nitrogen is indicative of severe poisoning, for this test is not very sensitive. Animals which showed an elevated NPN nearly always showed a proteinuria, catalasuria and amino aciduria.

It is of interest to compare the time course of each of the variables used in this study with that of the renal histopathology, which is presented in great detail elsewhere (13). Briefly, the course of histopathology is as follows: after about three days of exposure, degenerative changes occur in the proximal convoluted tubule, followed by necrosis and sloughing of dead cells into the lumen for about seven to ten days. The extent of the damage depends on the size of the exposure. Active regeneration starts within a few days after degenerative changes have first occurred and the tubules are completely relined in about two to three weeks.

After three to four weeks, the evidence of cell destruction is limited to occasional sloughed cells to the presence in some cases of atypical cells and some evidences of regenerative activity

The first appearance of albuminuria, catalasuria and amino aciduria seems to be definitely associated with the appearance of the first degenerative changes in the kidney. Thereafter, as the process of regeneration and repair is accomplished, the proteinuria and catalasuria tend to return to normal values. If azotemia results, it is at a peak after about seven to ten days which corresponds to the time at which maximal degeneration has occurred. The amino aciduria on the other hand remains elevated even after regeneration is essentially complete. Apparently the regenerated kidney, although normal in most other respects, cannot resorb amino acid nitrogen to the same extent as can the unpoisoned kidney.

#### SUMMARY AND CONCLUSIONS

1 The AAN/C ratio proved to be a very useful test for uranium poisoning in rabbits. The minimal dose of uranium that would elicit a significant response was 0.02 mgm of U/kgm of body weight given as uranyl nitrate. A proteinuria did not appear until 0.04 to 0.1 mgm of U/kgm were given.

2 With repeated intravenous injections of uranyl nitrate in rabbits at three to five-week intervals, certain patterns of response of AAN/C ratio were observed. The first response was always a marked peak followed by a return to lower but still abnormal values. In general, the magnitude of the response was proportional to the dose of uranyl nitrate given. After subsequent injections, the size of the peak usually diminished or even disappeared but the general level of the ratio remained abnormally high. With large doses of uranium, however, the ratio tended to become progressively higher with no disappearance of the peaks. The ratio did not return to normal for several months after the last injection even though the kidneys at this time showed no histopathology. The urinary protein tended to show a peak response after each injection followed by a return to normal.

3 During exposure to inhalation of uranyl fluoride dust, the urinary AAN concentration became elevated after three to five days and remained high throughout the rest of the 35 day exposure period. In contrast, the urinary protein, catalase and blood nonprotein nitrogen responses were more transient with high levels following the initial insult to the kidney followed by a return to more normal values after about two to three weeks when regeneration of damaged tubules in the kidney was almost complete.

4 The AAN response is independent of the protein and catalase response and for this reason, the three tests together are more sensitive than any one alone.

5 Some additional evidence is presented bearing on the development of tolerance by the kidney.

**ACKNOWLEDGEMENTS** The authors express their appreciation to Mr T B Barnett, Dr Frank Smith and Dr L Crump of the University of Rochester,



Atomic Energy Project. Mr. Barnett examined the histological sections; Dr. Smith's laboratory performed many of the biochemical tests on the inhalation experiments; and Dr. L. Crump supervised the statistical analyses.

#### REFERENCES

1. McNIDER, D. B.: *J. Exper. Med.*, **49**: 387, 1929.
2. DOUNCE, A. L., ROBERTS, E., AND WILLS, J. H.: *N.N.E.S.*, Div. VI, Book I, Vol. I, Chapter 14, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.
3. WILLS, J. H.: *N.N.E.S.*, Div. VI, Book I, Vol. I, Chapter 5, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.
4. DEVEZE, R.: *Compt. Rend. Soc. de Biol.*, **117**: 1111, 1934.
5. ROTHSTEIN, A., AND BERKE, H.: *THIS JOURNAL*, **96**, 179, 1949.
6. ROTHSTEIN, A., ET AL.: *N.N.E.S.*, Div. VI, Book I, Vol. I, Chapter 10, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.
7. PETERS, J. P., AND VAN SLYKE, D. D.: *Quantitative clinical chemistry*, Vol. I; 806, 1946, The Williams and Wilkins Co., Baltimore, Md.
8. FOLIN, O.: *Lab. Manual of Biol. Chem.*, 5th Ed., 1934.
9. ALBANESE, A. A., AND IRBY, I.: *J. Biol. Chem.*, **153**: 583, 1944.
10. HOFFMAN, W.: *Am. J. Clin. Path.*, **15**: 57, 1945.
11. FOLIN, O., AND WU, H.: *J. Biol. Chem.*, **38**: 81, 1919.
12. FLAGG, J. F.: *N.N.E.S.*, Div. VI, Book I, Vol. I, Chapter 2, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.
13. BARNETT, T. B., AND METCALF, R. G.: *N.N.E.S.*, Div. VI, Book I, Vol. I, Chapter 11, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.
14. VOEGTLIN, K., AND HODGE, H. C., ET AL.: *N.N.E.S.*, Div. VI, Book I, Vol. I, The pharmacology and toxicology of uranium compounds, McGraw-Hill Co., New York, 1949.

# PHARMACOLOGICAL STUDIES ON THE HYPNOTIC, 2-ETHYL-3-PROPYLGLYCIDAMIDE

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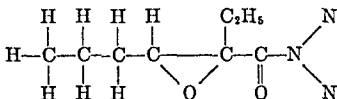
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Forneau, Billeter, and Bovet (1) reported that the 3,3-dimethyl, 3,3-diethyl, 3-benzyl-3-methyl, 3-phenoxyethyl-3-methyl, 3-cyclohexyl, 3-phenyl, 3,3-diethyl-N-methyl, and 3-phenyl-3-methyl-N-dimethyl derivatives of glycidamide all had weak hypnotic properties in experimental animals. Apparently none of these compounds were of sufficient interest to warrant clinical study.

Work on 2-ethyl-3-propylglycidamide<sup>2</sup> in this laboratory indicates this substance may prove to be of value clinically. It has a central depressant action in animals generally resembling that of the short-acting barbiturates.

2-Ethyl-3-propylglycidamide has the following structure.



It occurs as a tasteless white crystalline solid with little or no odor. One part is soluble in 95 parts of water at 30°C.

**EXPERIMENTAL.** *Hypnotic activity and acute intravenous toxicity in rabbits.* 2-Ethyl-3-propylglycidamide was compared to pentobarbital and ethylisoamylbarbituric acid employing the intravenous route in mature white rabbits. Comparative evaluations included determinations of the LD<sub>50</sub>, the M.H.D. (minimal hypnotic dose or the dose causing 50 per cent of the animals to lie on their sides with heads down), and the duration of action with physiologically equivalent doses of two M.H.D.s.

The three compounds were administered in equivalent amounts of a 50 per cent aqueous propylene glycol vehicle. The low solubility of the glycidamide in water necessitated the use of this solvent.

The vehicle itself killed 4 of 8 animals injected with amounts required to administer 360 mgm./kgm. (14.4 cc. of 50 per cent propylene glycol per kgm.) of the glycidamide and prevented the determination of an LD<sub>50</sub> for this substance. Smaller amounts of the propylene glycol vehicle required in other determinations had a negligible effect. The LD<sub>50</sub> values of 33 and 49 mgm./kgm. (table 1) for pentobarbital and ethylisoamylbarbituric acid in 50 per cent propylene glycol are in fair agreement with corresponding values of 40

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<sup>2</sup> Prepared in these laboratories by B. H. Harriman, present address: General Aniline and Film Corporation, Ansco Division, Binghamton, N. Y.; and G. H. Harris, present address: Dow Chemical Co., Pittsburgh, Calif.

*Chronic toxicity studies in rats.* The effects of daily stomach tube administration of 2-ethyl-3-propylglycidamide were investigated in three groups of rats. One group of six received 50 and one group of five 200 mgm./kgm. of the drug, suspended in 5 per cent gum acacia, six days per week for eight weeks. The third group of six animals served as a control and received daily administrations of 5 per cent gum acacia solution. Total volumes in all cases were 10 cc./kgm., and doses for individual animals were readjusted weekly to compensate for changes in body weight.

Cumulative narcotic effects did not develop even in the high dose series which received two-fifths of the M.H.D. daily; and signs of toxicity did not develop in either experimental group. Experimental and control animals gained in body weight at a similar rate. Red, white, and differential counts and hemoglobin concentrations remained in the normal range and did not differ significantly from the controls.

The histologic alterations in sections of the heart, aorta, lung, liver, spleen, stomach, adrenal, small and large intestine were inconsequential. They occurred as frequently in control as in experimental animals and thus could not be attributed to the administration of 2-ethyl-3-propylglycidamide.

*Chronic toxicity studies in monkeys.* One male and one female monkey (*Macaca mulatta*) received, by stomach tube, 100 mgm./kgm. per day of 2-ethyl-3-propylglycidamide, suspended in gum acacia, six days per week for four weeks.

Weight curves and hematologic findings for these animals were similar during a four-week pre-drug period, four-week drug period, and two-week post-drug period. Appetite, activity, and gross appearance of the animals remained normal throughout the period of observation, and there were no signs of toxic action of the glycidamide.

#### CONCLUSIONS

2-Ethyl-3-propylglycidamide is a short-acting hypnotic with central actions generally resembling those of the short-acting barbiturates.

Its milligram potency as determined in animals is roughly one-fifth to one-eleventh that of pentobarbital, one-fourth that of ethylisoamylbarbituric acid, and similar to that of  $\alpha$ -monobromoisovalerylurea. The margin of safety of the glycidamide is at least as large as that of the other hypnotics.

The duration of action is similar to that of pentobarbital, ethylisoamylbarbituric acid, and  $\alpha$ -monobromoisovalerylurea.

Repeated daily administrations of comparatively high doses of the glycidamide are nontoxic and nonaccumulative in laboratory animals.

#### REFERENCES

1. FOURNEAU, E., BILLETER, J.-R., AND BOVET, D.: J. Pharm. Chim., **19**: 49, 1934.
2. WERNER, H. W., PRATT, T. W., AND TATUM, A. L.: THIS JOURNAL, **60**: 189, 1937.
3. HUNT, H. W., FOSBINDER, R. J., AND BARLOW, O. W.: J. Am. Pharm. A. (Scient. Ed.), **35**: 244, 1946.
4. GRUBER, C. M., ELLIS, F. W., AND FREEDMAN, C.: THIS JOURNAL, **81**: 254, 1944.
5. HOLCK, H. G. O., AND KANAN, M. A.: J. Lab. & Clin. Med., **19**: 1191, 1934.
6. MILLER, L. C., AND TAINTER, M. L.: Proc. Soc. Exper. Biol. & Med., **57**: 261, 1944.

# THE PHARMACOLOGICAL PROPERTIES OF 2-METHYL,2-n-AMYL-4-HYDROXYMETHYL-1,3-DIOXOLANE (GLYKETAL), A NEW BLOCKING AGENT OF INTERNEURONS<sup>1</sup>

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Simple monoethers of glycerol have a peculiar depressant action on the central nervous system (1, 2) This depressant action differs from that of the barbiturates and other anesthetic drugs in not affecting consciousness and in having a greater effect on the mechanisms mediated in the spinal cord and midbrain than on those of the forebrain It was of interest to investigate whether other compounds containing the glycerol grouping would also exhibit similar depressant properties The first compound of this type which has been examined was the readily available glycerol ketal of cyclohexanone This substance in large doses appeared to possess a paralyzing action which on cursory examination was not dissimilar to that of the glycerol ethers Various derivatives of this compound were examined and it was found that the 2,2 alkyl-4-hydroxy methyl-1,3 dioxolanes possessed a paralyzing action which was quite similar to that observed with glycerol ethers (3) The present report deals with the pharmacological properties of 2 methyl, 2-n-amyl-4 hydroxymethyl 1,3 dioxolane which has been called Glyketal This compound possessed the greatest activity of all members of the series which have been examined

**I PHYSICAL AND CHEMICAL PROPERTIES** Glyketal is a colourless viscid liquid, possessing a very faint fruity odor, boiling point 128-132° at 10 mm pressure The compound is not entirely stable and hydrolyzes slowly on standing Glyketal is only very slightly soluble in water, about 0.1 per cent w/v It disperses easily in water or aqueous sodium bicarbonate solution and forms fairly stable emulsions A 0.5 per cent v/v emulsion is hazy and a 2 per cent v/v emulsion is of milky appearance A 0.1 per cent w/v sodium bicarbonate solution has been used as the dispersing medium to prevent hydrolysis This precaution is unnecessary if freshly prepared emulsions are used as hydrolysis did not occur for a few hours in emulsions made with distilled water

Glyketal and allied compounds were prepared by Drs V Boekelheide, D S Tarbell and their associates The chemistry of these compounds will be published by them elsewhere

**II PHARMACOLOGICAL PROPERTIES** The action of Glyketal was investigated in mice, rabbits, cats, and dogs The action was qualitatively similar in all these species and consisted in muscular relaxation and ataxia after small doses and muscular paralysis with impairment or loss of the righting reflexes after larger doses The paralyzing dose of Glyketal varied somewhat with the route of administration and the species of animals used The drug was however effective on

<sup>1</sup> Aided by a grant from The National Foundation for Infantile Paralysis.

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oral, subcutaneous, intramuscular, intraperitoneal and intravenous administration.

1. *Effect on mice.* Mice injected intraperitoneally with 150 to 200 mgm./kgm. became paralyzed a few minutes after administration of the compound. Paralysis was preceded by ataxia but excitation prior to paralysis was not observed or was slight. During paralysis the righting reflex was lost. The corneal, pinna and withdrawal flexor reflexes were present. Respiration was normal or slightly increased in rate and depth. Paralysis with these doses lasted a few minutes and was soon followed by complete recovery of voluntary muscular function. Higher doses of Glyketal caused paralysis of longer duration. Lethal doses appeared to cause death by respiratory paralysis. The effects observed with Glyketal in mice could not be distinguished from those obtained after injection of comparable doses of Myanesin.

The relative efficacy and toxicity of Myanesin and Glyketal has been compared in mice by giving graded doses of both compounds under similar conditions on the same day to groups of male animals of similar weight. The results are

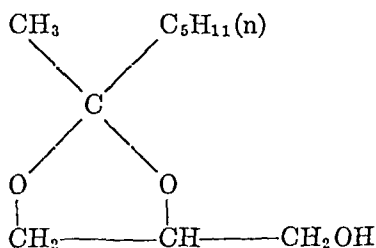


FIG. 1. THE STRUCTURAL FORMULA OF 2-METHYL, 2-N-AMYL-4-HYDROXYMETHYL-1,3-DIOXOLANE (GLYKETAL).

summarized in table 1. Glyketal appeared to be a more effective paralyzing agent than Myanesin. The difference between the mean paralyzing doses of the two compounds was statistically significant ( $P < 0.01$ ). Glyketal was also somewhat less toxic than Myanesin but the difference may well have been due to chance ( $P$  about 0.1). The standard safety margin calculated according to Foster (4) as the percentage above the surely effective dose at which an occasional death will occur was 78 per cent for Glyketal and 58 per cent for Myanesin.

The duration of paralysis obtained with the two compounds has been compared by the cross over technique after a dose of 180 mgm./kgm. Every animal received both drugs at an interval of four days. The mean duration of paralysis with Glyketal was 6 min. 48 sec.  $\pm$  1 min. 6 sec. as compared with 1 min. 23 sec.  $\pm$  38 sec. for Myanesin.

The results of these experiments indicate that Glyketal possesses greater paralyzing power and that it causes paralysis of longer duration than Myanesin. The toxicity of both compounds in mice appears to be similar.

2. *Effect on rabbits.* The effect of an intravenous injection of Glyketal to rabbits varied with the rate of injection. When injections were carried out at the

rate of 40 mgm /kgm /min the animals showed ataxia soon after termination of the injection. The movements of the head and extremities were imperfectly controlled. This stage was followed after about two minutes by impaired muscular power and general muscular flaccidity. The ears drooped, the head could not be held up and the body sank down and spread out along the ground. Muscular power was, however, not entirely lost as the animals were still able to react vigorously to stimulation. Certain rabbits showed vertical nystagmus for a short period of time. The corneal and pupillary reflexes and the knee jerk were present. Animals placed on their side remained in this position for five to fifteen minutes. Respiration was unchanged or slightly increased in rate and depth.

TABLE 1

*The incidence of paralysis and death after intraperitoneal administration of Glyketal and Myanesin to white male mice, weighing 18-20 grams*

The numerator gives the number of animals reacting and the denominator the number of animals used

MGm /kgm	GLYKETAL		MYANESIN	
	Paralysis	Death	Paralysis	Death
120	4/20	0/20	0/20	0/20
150	6/20	0/20	0/20	0/20
180	18/20	0/20	7/20	0/20
230	20/20	0/20	18/20	0/20
280	20/20	0/20	20/20	0/20
420	40/40	4/40	20/20	0/20
520	20/20	4/20	20/20	7/20
620	40/40	23/40	20/20	19/20
ED <sub>50</sub>	148 ± 6 mgm /kgm		191 ± 10 mgm /kgm	
LD <sub>50</sub>	595 ± 26 mgm /kgm		540 ± 27 mgm /kgm	
LD ED <sub>50</sub>	4.0		2.8	
Standard safety margin	78%		58%	

During this stage the animals appeared conscious and followed moving objects with the eyes. On recovery from paralysis the animals sometimes closed their eyes and appeared to fall asleep for a few minutes. All animals receiving doses of this order recovered and remained well. Doses of 80 mgm /kgm /min caused complete flaccid paralysis lasting for about twenty minutes. Soon after the termination of the injection the animal became incapable of any movement and did not react to stimuli except by a slight narrowing of the palpebral fissure. The limbs were flaccid. The corneal reflex was usually absent for a short time during the first few minutes of paralysis. Respiration was shallow and somewhat decreased in rate but the animals continued breathing spontaneously. Recovery of muscular power in the neck muscles and fore limbs always occurred some minutes before muscular power returned to the hind legs. The animals were com-

pletely recovered one to two hours later. They sometimes died overnight for unknown reasons.

Larger doses of the drug or similar doses injected more rapidly in stronger solution caused death by respiratory paralysis. The heart continued beating for a minute or so after respiration had stopped. On post-mortem examination after administration of lethal doses the right heart was as a rule well filled, the liver was congested and there were numerous hemorrhages in both lungs.

Glyketal never caused excitement prior to paralysis. The occurrence of rigors as described after rapid intravenous doses of Myanesin to rabbits (5) has not been observed after Glyketal. Extensor rigidity of the decerebrate type, however, has been observed after rapid intravenous injection of Glyketal to cats.

3. *Effects on cats.* The intraperitoneal administration of Glyketal to cats in doses of 100 mgm./kgm. caused generalized muscular weakness. A few minutes after administration of the drug the animals were unable to move around in a coordinated manner. The ataxia was probably due to both impaired muscular power and a disturbance of equilibrium. About ten minutes after injection the animals were unable to support the body on the legs and lay on their side. They were, however, capable of movement on stimulation. During paralysis which lasted 40 minutes or longer respiration and the corneal and deep reflexes were normal. When recovery set in disturbances of equilibrium again became apparent for half an hour or so. Sometimes a transient tremor of the hind legs was observed. In these animals the administration of the drug was repeated on three successive days with similar result. Untowards after-effects were not observed during two weeks observation.

The animals did not show signs of excitation prior to or following paralysis. Vomiting was not observed. Specimens of urine collected at various times after administration of the drug did not contain abnormal constituents.

4. *Effect on circulation and the autonomic nervous system.* These effects were investigated in cats anesthetized with Dial solution 0.6 cc./kgm. The intravenous injection of Glyketal 5 mgm./kgm. did not affect respiration but produced a slight and transient fall of blood pressure and a decrease in heart rate. Smaller doses did not cause significant effects. As was the case with Myanesin, the depressor effect obtained depended to a large extent on the speed of injection. A rapid injection of 10 to 20 mgm./kgm. produced marked hypotension which was, however, of short duration and followed by spontaneous recovery. Similar amounts injected very slowly had hardly any effect on blood pressure.

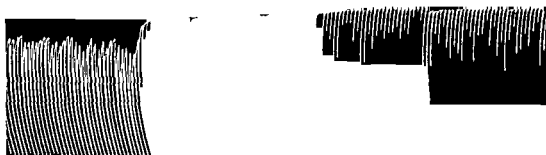
Glyketal did not appear to have any muscarinic effect as the fall of blood pressure and slowing of the heart were not altered after the administration of atropine. The drug also appeared devoid of nicotinic action. The repeated administration of a dose caused a depressor effect of comparable extent.

The blood pressure responses to epinephrine, acetylcholine and histamine were not altered even after large doses of Glyketal. The drug did not influence the depressor effect and slowing of the heart produced by stimulation of the peripheral vagus nerve.

5. *Effect on neuromuscular transmission.* The effect of Glyketal on neuromuscular transmission and spinal reflexes was examined in cats anesthetized with

Dial solution 0.6 cc /kgm. The gastrocnemius muscle was stimulated through its nerve by a series of short tetani administered from a Harvard inductorium every 10 seconds. The duration of each stimulus was about 100 milliseconds. The contractions of the muscle were recorded on a smoked drum. Glyketal was injected intravenously into the jugular vein. It did not affect the excitability of the muscle to indirect stimulation in doses up to 20 mgm /kgm. This lack of any curare-like action of Glyketal is illustrated in fig. 2. d-Tubocurarine chloride administered similarly in doses of 0.06 mgm /kgm caused marked but transient reduction of excitability of the muscle to indirect stimulation.

#### FLEXOR REFLEX



#### INDIRECT STIM

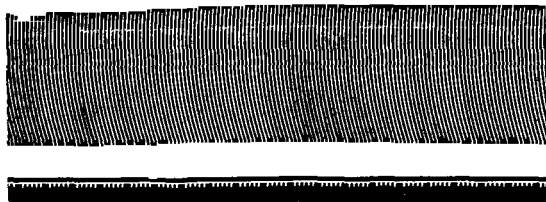


FIG. 2 THE EFFECT OF GLYKETAL ON THE FLEXOR REFLEX AND STIMULATION OF THE GASTROCNEMIUS MUSCLE THROUGH ITS NERVE

Cat 2250 gm. Dial anesthesia. Tracings from above downward: Flexor reflex, indirect stimulation, signal line and time in 10 second. At signal intravenous injection of Glyketal 30 mgm.

6 *Effect on spinal reflexes* The knee jerk was elicited mechanically every 10 seconds with a solenoid tapper of the type used by Schweitzer and Wright (6). The tibialis anticus muscle was used for the recording of the flexor reflex. After section of the ipsilateral femoral nerve and the hamstring branch of the sciatic nerve, the posterior tibial nerve, severed distally to the electrodes, was stimulated. The stimuli were derived from a Grass stimulator and consisted of short tetani applied for about 100 milliseconds at 10 second intervals. The drugs were injected into the external jugular vein.

Glyketal as a rule did not affect the normal knee jerk in any way. Sometimes



there was a slight decrease in the size of the jerk. This effect may have been due to the general diminution of muscular tone by Glyketal. Complete inhibition of the knee jerk with tolerated doses of Glyketal was never observed.

The effect of the drug on the flexor reflex was very marked (fig. 2). Small doses of Glyketal of the order of 10 mgm./kgm. caused a depression or complete disappearance of the flexor reflex. The effect was almost immediate and was of fairly long duration. Myanesin possessed a qualitatively similar action which was, however, of lower intensity and shorter duration. To obtain similar effects with the two drugs, about twice as much Myanesin as Glyketal had to be used.

### CROSSED EXTENSOR REFLEX

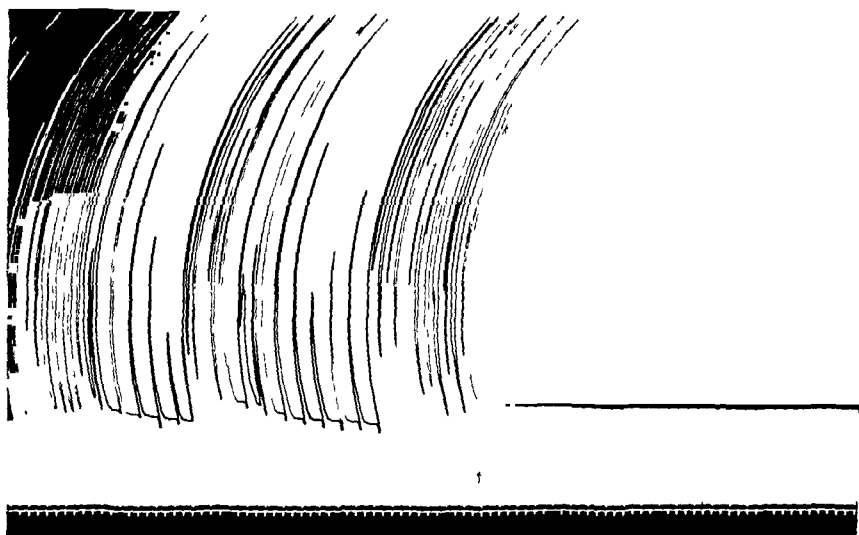


FIG. 3. THE EFFECT OF GLYKETAL ON THE CROSSED EXTENSOR REFLEX

Cat 2640 gm. Dial anesthesia. Tracings from above downward: Crossed extensor reflex, signal line marking stimulation and time signal every 5 seconds. At arrow injection of Glyketal 15 mgm.

Glyketal also had a marked depressant action on the crossed extension reflex produced by stimulation of the central stump of the contralateral sciatic nerve. A series of these reflexes is shown in fig. 3. Although the strength and duration of the stimulus was the same in every case, the reflex showed marked variations in response. A series of five or six reflexes showing marked after-discharge was followed by a series of four or five reflexes with little after-discharge. These groups formed a fairly regular pattern. Glyketal abolished the crossed extensor reflex regularly. This reflex was most sensitive to the effects of the drug and could be abolished for long periods of time with very small amounts of Glyketal.

Although Glyketal had but little effect on respiration in doses which depressed the flexor and extension reflexes, the possibility that the depression of reflexes was caused by anoxia was considered. It was found that forced artificial respiration

administered under positive pressure did not affect the effect of Glyketal on the reflexes in any way

7 *Effect on reflex hyperexcitability* A state of increased reflex hyperexcitability was produced in anesthetized cats by the administration of suitable doses of strychnine. A repetitive stimulus which kept excitation at a relatively high and constant level was provided by tapping the quadriceps tendon at intervals of 5 seconds with an automatic tapper. With this technique a tremor of the stim-

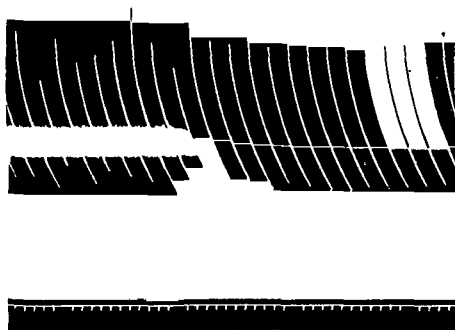


FIG. 4 THE EFFECT OF GLYKETAL ON THE KNEE JERK AND ON TREMOR PRODUCED BY STRYCHNINE

Cat 2620 gm. Dial anesthesia. Strychnine sulphate 0.5 mgm. administered in divided doses. Tracing from above downward: knee jerk, signal line and time in 5 second intervals. At signal Glyketal 30 mgm. intravenously.

ulated leg was often produced for prolonged periods of time. Because of the tremor the size of the knee jerk was irregular. Injection of Glyketal almost immediately abolished the tremor and permitted the eliciting of a normal knee jerk. This effect is illustrated in fig. 4. The broad white line was produced by the rapid oscillation of the lever due to the tremor. After administration of the drug the tremor stopped completely as evidenced by the narrow trace between the tendon reflexes.

On other occasions administration of strychnine did not produce tremor but

caused the appearance of periods of hyperexcitability which appeared fairly regularly after every five to seven knee jerks. Administration of Glyketal abolished this repetitive pattern of excitation but did not depress the knee jerk. An example of such a record is given in fig. 5.

8. *Drug antagonism.* The antagonistic action of Glyketal and Myanesin to the effects of strychnine, pentamethylenetetrazol (Metrazol) and Picrotoxin was investigated in white male mice weighing 18-20 grams. The convulsant drugs

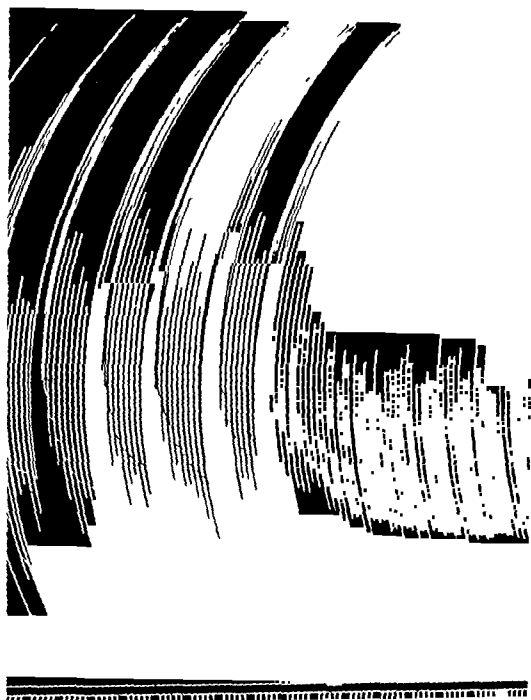


FIG. 5. THE EFFECT OF GLYKETAL ON THE KNEE JERK IN A STRYCHNINIZED CAT

Cat 2800 gm. Dial anesthesia. Strychnine sulphate 0.6 mgm. given in divided doses. Tracings from above downward: knee jerk, signal line and time signal every 5 seconds. At signal Glyketal 30 mgm. intravenously.

were administered in doses which caused convulsions and death in the majority of the animals ( $LD_{50}$ ). Glyketal or Myanesin were administered simultaneously with one of the convulsants by the intraperitoneal route. Ten mice were used at each dose level. The volume of fluid which was injected was kept constant. The incidence of convulsions and deaths was noted at intervals of 10 minutes for 90 minutes. The results of the experiment are summarized in table 2.

Glyketal possessed some antagonistic action to the lethal effects of strychnine and Metrazol but this action was much weaker than that of Myanesin. The difference between the antagonism of the two depressant drugs to Metrazol was of

particular interest. Large paralyzing doses of either drug were able to prevent both convulsions and death from Metrazol. With smaller doses Glyketal behaved as a true, though weak anticonvulsant by showing a comparable effectiveness in preventing both convulsions and death. Myanesin on the other hand was very effective in preventing death but had little effect on the incidence of convulsions. Control animals receiving Metrazol only, usually died a few minutes after the onset of convulsions while the mice which also received Myanesin convulsed violently for one or two hours but did not die. The convulsions in the Myanesin treated animals appeared just as violent as those of the control.

TABLE 2

*The antagonistic action of Glyketal and Myanesin to the lethal effects of strychnine, Metrazol and Picrotoxin in white male mice on intraperitoneal injection*

An LD<sub>50</sub> dose of one of the convulsants was injected jointly with graded doses of Glyketal or Myanesin and the incidence of death noted at various times after injection. Ten mice were used at each dose level.

CONVULSANT	TIME AFTER INJECTION	CONTROLS No died/No injected	NUMBER OF DEATHS AFTER					
			Glyketal mgm /kgm			Myanesin mgm /kgm		
			250	125	62.5	250	125	62.5
Strychnine 2 mgm /kgm	10	16/20	0	4	6	0	1	8
	30	17/20	2	7	7	0	3	8
	60	17/20	2	8	8	0	4	8
	90	17/20	3	8	8	0	4	8
Metrazol 100 mgm /kgm	10	15/15	0	1	5	0	0	2
	30	15/15	0	9	10	0	0	4
	60	15/15	6	10	10	1	3	5
	90	15/15	10	10	10	1	4	6
Picrotoxin 15 mgm /kgm	10	0/20	0	0	0	0	0	0
	30	15/20	4	7	10	0	4	8
	60	16/20	7	8	10	3	7	9
	90	16/20	9	8	10	5	10	9

9 *Other pharmacological properties* Glyketal did not possess analgesic action in doses not causing paralysis. During paralysis the animals did not react to painful stimuli of moderate intensity but this was probably secondary to the muscular impairment. Reactions to strong stimuli were present during paralysis. Glyketal when tested on the rabbit's cornea had a local anesthetic action comparable to that of procaine. The local anesthetic action was of little practical interest because of the irritation caused by the compounds.

DISCUSSION. The experiments described in this paper suggest that Glyketal exerts its effects by a highly selective depressant action on certain parts of the central nervous system. The effect of the drug on certain spinal reflexes permits the identification of the locus of action of the drug. Recent work (7) has shown that the knee jerk is mediated by a two neuron reflex arc formed by the direct

synapse between the large afferent nerve fibres and the anterior horn motor cells. The flexor reflex on the other hand is mediated through a three-neuron or multi-neuron arc in which one or more interneurons in the spinal cord, are interposed between the afferent nerve-ending and the anterior horn motor cell. In the case of the crossed extension reflex with its long latency and prolonged after-discharge several interneurons are interposed between the afferent and efferent parts of the reflex arc. Administration of Glyketal in small doses suppresses the crossed extension reflex for long periods of time. Somewhat larger doses of the order of 10 mgm./kgm. depress or abolish the flexor reflex but do not influence the knee jerk. There appears to be a relation between the effect of Glyketal and the presence of interneurons in the reflex arc. If interneurons are not present in the reflex arc as in the case of the knee jerk the drug does not influence the normal reflex happenings. If one interneuron is intercalated in the reflex arc the depressant effect on the reflex is marked and when several interneurons are interposed the depressant effect of the drug becomes very strong. The drug also appears effective in suppressing the central excitatory state produced by the injection of strychnine and sustained by elicitation of the knee jerk at regular intervals. The maintenance of the central excitatory state has been explained by the activity of interneurons (8). All of these results suggest that Glyketal blocks the spread of impulses through interneurons of the spinal cord.

Apart from this action Glyketal in small doses seems to have little effect on the workings of the nervous system. On systemic administration it does not affect conductivity of nerves and does not influence the transmission of impulses at the myoneural junction. Electroencephalographic studies carried out with three cats showed no evidence of significant alterations in the electrical activity of the cortex after Glyketal in doses of 20 mgm./kgm.

Two recently described drugs, benzimidazole (9) and Myanesin (1) which are chemically unrelated to each other and to Glyketal also have a depressant action on interneurons. They differ from Glyketal in being weaker interneuronal blocking agents and in possessing a much stronger antagonistic action to strychnine.

A depressant action on interneurons is also an important property of many general anesthetics such as the barbiturates. These anesthetic drugs differ, however, from benzimidazole, Myanesin and Glyketal in influencing consciousness first and affecting the interneurons in larger doses only. Glyketal and the other two agents depress the interneurons in doses which do not cause paralysis and do not seem to affect consciousness.

The loss of the righting reflex, the disturbances of equilibrium and the nystagmus observed after larger doses of Glyketal indicate that the medulla oblongata and the midbrain are also affected. These effects may also be due, at least in part, to interneuronal blockage. There is, however, the possibility that Glyketal also exerts direct depressant effects on neurons mediating postural and optic reflexes.

A drug which can produce muscular relaxation and reduce reflex hyperexcitability in doses which do not affect respiration or consciousness is obviously of potential interest in therapeutics. Glyketal is likely to be of value in conditions

in which spasm and pain are caused or mediated by abnormal activity of the interneurons Glyketal may also temporarily abolish involuntary movements maintained by circulation of impulses in closed interneuronal circuits Apart from any value that the drug may have in therapeutics it may become, because of its selective action, a valuable tool in neurophysiological studies

#### SUMMARY

2-Methyl, 2-n-amyl-4-hydroxymethyl-1,3-dioxolane, called Glyketal, produced transient diminution of muscular tone and paralysis of voluntary muscles in doses which did not markedly affect respiration and other vital functions In small doses which did not cause paralysis Glyketal had a marked depressant action on the flexor and crossed extensor reflexes The normal knee jerk was not affected The drug counteracted the central excitatory state produced by strychnine These results indicate that Glyketal has a selective blocking action on the interneurons of the spinal cord

Acknowledgment My thanks are due to Dr R Plato Schwartz for his interest in this work I am also indebted to Drs V Boekelheide, D S Tarbell and their associates for supplies of Glyketal, to Mr D E Leary and Miss Anna-mae Wolf for technical assistance and Mr Tyler Tinker for the photographs

#### REFERENCES

- 1 BERGER, F M , AND BRADLEY, W Brit J Pharmacol , 1: 265, 1946
- 2 BERGER, F M THIS JOURNAL , 93, 470, 1948
- 3 BERGER, F M , BOECKELHEIDE, V , AND TARBELLI, D S Science, 108: 561, 1948
- 4 FOSTER, R H K THIS JOURNAL , 65: 1, 1939
- 5 BERGER, F M Brit J Pharmacol 2: 241, 1947
- 6 SCHWEITZER, A AND WRIGHT, S J Physiol , 88, 459, 1937
- 7 LLOYD, D P C J Neurophysiol , 6 293, 1943
- 8 LORENTE DE NO, R J Neurophysiol , 2 402, 1939
- 9 GOODMAN, L Bull New England Med Center, 5: 97, 1943

# DEPOSITION OF RADIO GALLIUM ( $\text{Ga}^{72}$ ) IN SKELETAL TISSUES<sup>1</sup>

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Findings of studies on the metabolism of gallium (1, 2, 3) indicated that in this element there existed a new tool for the study of bone metabolism. On the basis of the information and experience gained in this work an investigation was initiated covering the physiological characteristics of the radioactive isotope ( $\text{Ga}^{72}$ ), which is an energetic beta and gamma emitter having a half life of 14.1 hours.

In this report are given the results of a radioautographic study of the distribution of gallium in the skeletal tissues of the rabbit and dog following injection of a non-toxic dose of radiogallium ( $\text{Ga}^{72}$ ).

**PREPARATION OF RADIOAUTOGRAPHS.** The radioautographs shown in figures 1 and 2 were prepared by injecting  $\text{Ga}^{72}$  as the lactate or citrate into rabbits and dogs at a dosage level of 0.5 millicuries/kgm. body weight. The animals were killed twelve hours after injection and the bones removed and cleaned free of adhering tissue.

For the gross sections the bones were immediately mounted in quick setting dental stone, and the mounting allowed to harden about twenty minutes. The mount was then sectioned in layers at least fifteen mm. thick using a fine toothed band saw. The sections after being smoothed and flattened on emery paper were suitable for mounting on the photographic film. A metal plate 5" x 7" was overlaid with paper and a section of single emulsion X-ray film was placed on this paper. Over the emulsion was laid a sheet of aluminum foil (.025 mm.) and the mounted sections placed on the foil. The whole was bound tightly with scotch tape, wrapped in black paper and allowed to remain for three to four hours. The film was then removed and developed six minutes at 20°C. in X-ray film developer.

The thin sections (fig. 1, c, fig. 2, c, d) were prepared by sectioning the fresh tissue by means of a diamond wheel or carborundum disc to a thickness of 2 mm. This section was dehydrated in absolute ethyl alcohol and attached with some suitable cement to a cork mounting. The specimen was then dry ground to about .05 mm., washed with alcohol dried, and placed in direct contact with the emulsion of a single emulsion X-ray film for 24 to 48 hours. More details of the procedure will be given later (5).

For the radioautographs shown in this report rabbits and young dogs were used because of the ease of handling and preparation. Other species offer no particular difficulties except when using small animals, the bones tend to shatter on sectioning with a band saw. For these smaller specimens a thin emery wheel such as used for glass cutting has been found satisfactory.

The radiogallium as administered was contained in stable carrier gallium so that at a level of 0.5 mc/kgm. a total dosage of 8 mgm. Ga/kgm. resulted. This level of the radioactive isotope has produced no observable effects when administered to dogs or rabbits. Gallium may be given as the lactate (4) intravenously. When administered subcutaneously the citrate is more satisfactory, particularly if some local anesthetic is used at the site of injection.

<sup>1</sup> The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Navy Department.

**RESULTS** In figure 1 are shown contact prints of the radioautographs prepared from representative skeletal tissues of the rabbit. As will be seen in (a) the epiphyseal junction of the femur is clearly outlined. This bone is from a young animal (1.5 kgm) and indicates the selectivity with which gallium is deposited in those areas of greatest osteogenic activity. In grown animals there is also a deposition in this general area but it takes place in a more diffuse manner throughout the trabecular bone. In figure 1 (c) (d) the deposition in the osteoid structure of the spine is demonstrated. In (e) (f) the deposition in the peridonta

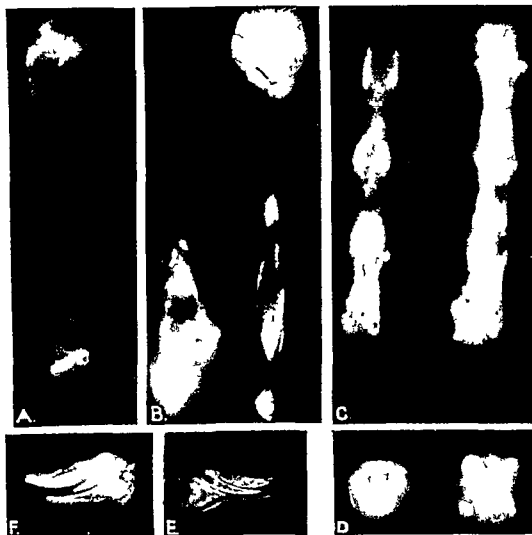


FIG. 1. RADIOAUTOGRAHS OF SECTIONS OF SKELETAL TISSUES OF RABBIT

(a) longitudinal of femur, (b) oblique of femur, (c) longitudinal of spine, (d) transverse of spine, (e) thin section of mandible and incisor, (f) thick section of mandible and incisor

and tooth pulp is shown. In these illustrations the dentine appears only as black areas, since this tissue does not absorb the gallium within twelve hours. More detailed studies of the dental phases of the problem are to be reported (5).

In figure 2 (a) (b) the deposition of radiogallium is shown in the bones of a two months old dog (4.6 kgm) indicating again the rapidity with which gallium enters those areas of greatest osteogenic activity. It should be noted that the entry of gallium into the cortical bone is here clearly indicated, since the shaft of each long bone is easily observed. This observation confirms the earlier finding, by chemical analysis, that the entry of gallium into cortical bone is an unusually dynamic process (1, 3).



The thin sections of bone (c) (d) produced radioautographs which give a more detailed picture of the site of deposition of the gallium in the ends of the long bones. In (d) the epiphyseal line is clearly evident but some Ga is also deposited in the adjacent trabecular bone. The radioautograph (negative of these thin sections) is of sufficient detail to permit magnification at low power. The contact prints as seen here do not carry the detail this clearly.

DISCUSSION. In many classical experiments strontium has been shown to substitute for or replace calcium in low calcium or rachitic diets. This process is one of moderate speed and depends in large degree on the usual body mechanisms

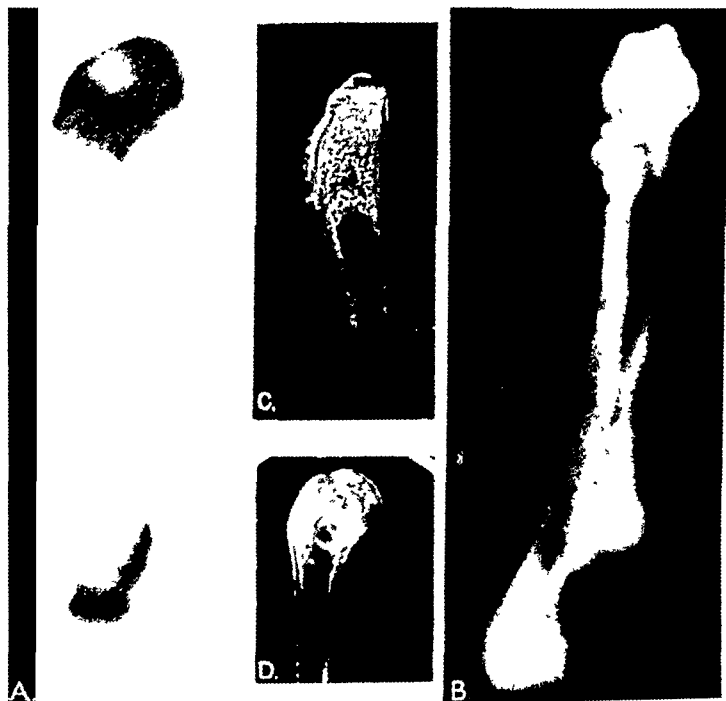


FIG. 2. RADIOAUTOGRAHS OF SKELETAL TISSUES OF DOG AND RABBIT

(a) femur of dog, (b) articulated foreleg of dog, (c) (d) thin sections from long bones of rabbit.

for the deposition of calcium. However, the speed of entry of gallium into cortical bone indicates that by the use of this element, we may open up new avenues of approach to the study of bone metabolism, independent of the mechanisms responsible for calcium deposition.

The most significant finding in this series of radioautographs is the rapidity of deposition of gallium and marked radioactivity at that area of greatest osteogenic activity, namely the epiphyseal junction in the young animal. On the basis of these findings a program for the study of the effect of radiogallium on osteogenic neoplasms has been initiated. This study will include effects on induced as well as spontaneous tumors.

## SUMMARY

Radioautographs of bones of rabbits treated with radiogallium ( $Ga^{72}$ ) strengthen the hypothesis that gallium is a useful tool for the study of bone metabolism. Gallium is deposited throughout the osteoid tissues of the rabbit and dog. It is especially concentrated at these areas of greatest osteogenic activity in the young animal, i.e., the epiphyseal line.

## REFERENCES

- 1 DUDLEY, H. C. THIS JOURNAL, 95: 482, 1949
- 2 DUDLEY, H. C., AND LEVINE, M. D. THIS JOURNAL, 95: 487, 1949
- 3 DUDLEY, H. C., MADDOX, G. F., AND LARUE, H. C. THIS JOURNAL, 96: 135, 1949
- 4 DUDLEY, H. C., AND GARZONI, R. F. J. Am. Chem. Soc., 70: 3942, 1948
- 5 ENGLISH, J. A., AND DUDLEY, H. C. In Preparation

# VASO- AND BRONCHODILATOR EFFECTS OF N-ISOPROPYL-NOREPINEPHRINE IN ISOLATED PERFUSED DOG LUNGS

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Evidence of the bronchodilator action of N-isopropyl-norepinephrine<sup>2</sup> (proprietary names: Aludrin, Aleudrin, Aleudrine, Isuprel, Neo-Epine, Neodrenal, Norisodrine) was first obtained from experiments on anesthetized dogs and guinea pigs (Konzett, 1, 2). This finding has since been confirmed for the cat and for the guinea pig by other workers using the method designed by Konzett and Rössler (3) and other methods (Fromherz, 4; Lissak *et al.*, 5; Hamburger *et al.*, 6; Lands *et al.*, 7; Siegmund *et al.*, 8; Marsh *et al.*, 9). The action of N-isopropyl-norepinephrine (I-a in the following account) on the systemic blood pressure has also been investigated and it has been found to have a vasodepressor action due to peripheral vasodilatation (Konzett, 10; Lands *et al.*, 7; Marsh *et al.*, 9). Until now, however, no experiments have been carried out to show what its action may be on the lesser circulation. It has seemed advisable to investigate this question since the possible effects of the drug on the pulmonary blood vessels may be of importance in connection with its clinical use. Such an investigation can only be carried out under conditions which exclude cardiac and systemic effects, which otherwise may complicate measurements of resistance or capacity changes in the pulmonary vessels, since I-a has a strong stimulating action on the heart in addition to its vasodilator action (Konzett, 10; Domini, 11; Lissak *et al.*, 5; Lands *et al.*, 7; Marsh *et al.*, 9).

For this purpose the isolated perfused dog lungs may conveniently be employed. The preparation has the further advantage that either negative or positive pressure ventilation may be used.

**METHODS.** The experiments were carried out on isolated dog lungs perfused with blood. Tests were made under three different conditions of experiment, each of which involved somewhat different procedures in the preparation. These were as follows:

1. Isolated perfused lungs prepared according to a technique modified from that originally described by Daly (12) for *Macacus rhesus*. Instead of defibrinating the blood, heparin was used as an anticoagulant, being given intravenously (2-3 cc. Roche Liquaemin) before death as well as being added to the blood as it was shed (ca. 0.5 cc. per 100 cc. blood). A second modification was that the lungs and heart were removed from the chest after ligation of all vascular connections to other structures. Having administered heparin to the animal before death it was not considered necessary to wash the pulmonary vessels with blood as a preliminary to perfusion.

2. Isolated perfused lungs, the dissection of which was carried out sufficiently far on the living animal during chloralose anaesthesia (0.1 gm./kgm.) so that the further steps necessary to start perfusion occupied only 1-2 minutes from the time that death occurred. Heparin was again used as an anticoagulant. The lungs were left in the thorax until after perfusion had been begun; they were then removed as perfusion continued and were trans-

<sup>1</sup> British Council Scholar.

ferred to the respiratory chamber. The volume of blood required for perfusion in this and the previous method was approximately 300—400 cc.

3. Separate perfusion of the right and left lungs to provide two preparations on which tests could be made independently. The method was that described by Daly *et al.* (13) and later modified by Hebb and Nimmo Smith (14). With this technique the volume of blood used for perfusing each lung was 300 cc, making a total of 600 cc for both circuits.

Results obtained with the three different methods of experiment did not differ from one another significantly.

**Blood Circuit.** In all experiments the lungs were perfused through the pulmonary artery by means of a Dale Schuster pump. When the circuit was closed, as was the case when the first or second method was used, the blood from the lungs was re-collected through a cannula in the left auricle and returned to the venous reservoir from which the pump was fed. In such experiments continuous records (kymographic) were taken of (a) the pulmonary arterial pressure (P A p) and (b) the venous reservoir volume (V R). When an open circuit was used, and this was obligatory with the separated isolated lungs (third method), the blood returning from each lung was allowed to drip from the opened veins into a reservoir which was in turn connected to the pump from which that lung was fed. In such experiments the P A p but not the V R was recorded. Records of the P A p were obtained by means of a small tambour (Palmer) calibrated in cm of blood. The venous reservoir volume was measured by means of a float recorder (Palmer). It should be noted that a rise or fall in the V R indicates an equal but opposite change of the lung blood volume (12).

**Ventilation.** The respiratory chamber when closed formed a rigid box which, with the exception of the glass cover (sealed on tightly with high melting point vaseline), was made of heavy aluminum. A connection from the chamber led to a vacuum cleaner in order to produce a negative pressure which for the purpose of ventilation could be rhythmically abolished by means of a valve opening and closing at the rate of 12—13 times per minute. With this method of ventilation the tidal air volume (T A) was recorded on the kymograph by means of a spirometer (350 cc capacity). In the case of the separated perfused lungs a pair of smaller spirometers (each 250 cc in capacity) was used.

For positive pressure ventilation a Starling Ideal pump was employed. By an arrangement similar to that devised by Konzett and Rossler (3) the air was delivered from the pump to the lungs at constant pressure (100—150 mm. water) and the excess volume not taken into the lungs measured by means of a piston recorder. Thus with bronchodilatation, the excursion of the piston recorder was diminished, the excursion increased when bronchoconstriction occurred. The apparatus was so arranged that it was possible to change over quickly from negative to positive pressure ventilation and *vice versa*. Whether negative or positive pressure ventilation was used the results obtained were substantially the same.

Drugs were most frequently administered by injection into the blood inflow tubing, the volume of each injection varied between 0.1—0.5 cc. Occasionally drugs were prepared in the form of a spray (produced by a Collison inhaler, 8 l. oxygen per minute) and administered by inhalation. When using this method a Siche Gorman two way valve was attached to the trachea and the spray was administered from a rubber bag attached to the inspiratory side of the valve. This method was used only when the lungs were under negative pressure ventilation. Measurements of the expiratory volume for one or two respiratory cycles could be made from time to time by connecting a spirometer to the expiratory side of the valve.

Drugs tested were N-isopropyl norepinephrine sulphate (manufactured by C. H. Boehringer Sohn, Ingelheim a. Rh.), epinephrine (Pike Davis solution), histamine acid phosphate (BDH), pilocarpine HCl (Harkness, Beaumont and Co., Edinburgh), acetylcholine (Roche), ergotamine ethanesulphonate (BDH), ergotamine and dihydroergotamine (Sandoz).

**RESULTS.** *Pulmonary vasomotor responses to N-isopropyl norepinephrine* I-a, in doses of 1 microgm. or more, consistently caused a slight but definite fall in pulmonary arterial pressure (fig. 1). The decrease in resistance was some-

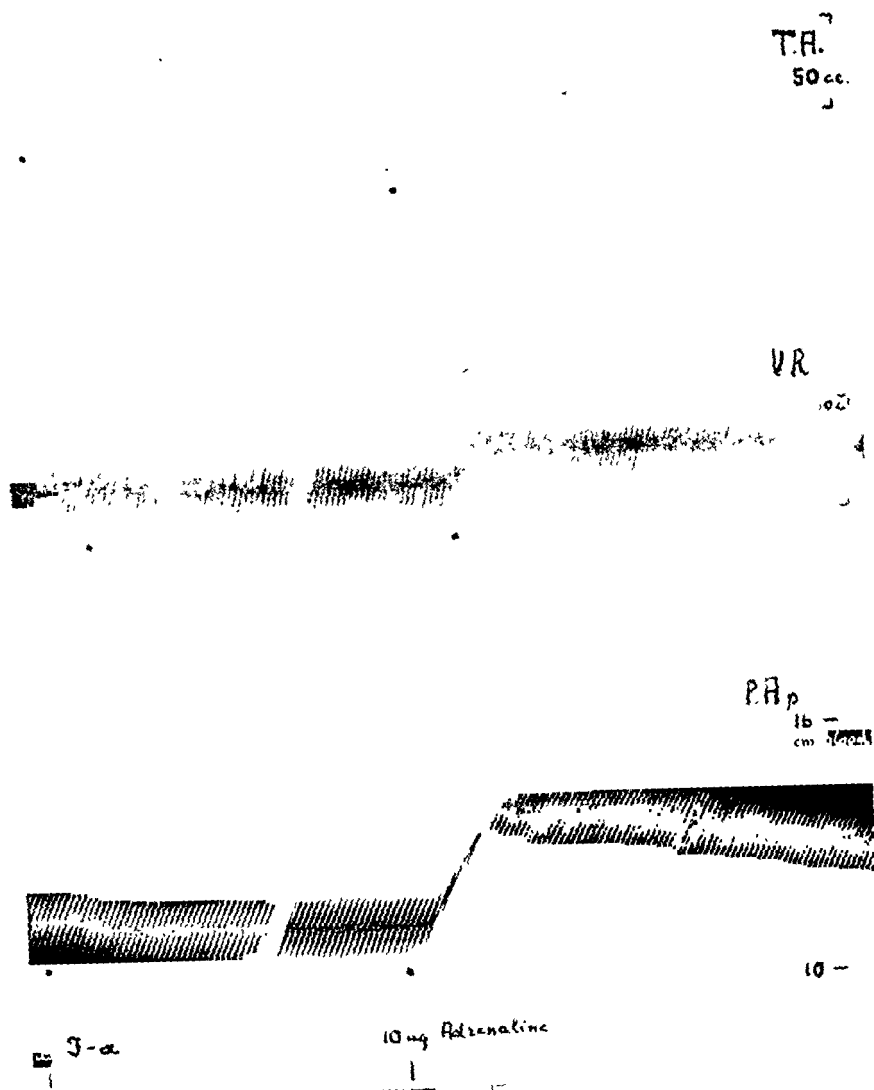


FIG. 1. I.P.L. (ISOLATED PERFUSED LUNGS)

Dog, 11.3 kgm., showing the effect of injections of 1 microgm. N-isopropyl-norepinephrine (I-a) and of 10 microgm. epinephrine on tidal air (T.A.), venous reservoir volume (V.R.), and pulmonary arterial pressure (P.A.p.). Times in this and other figures: 1 minute.

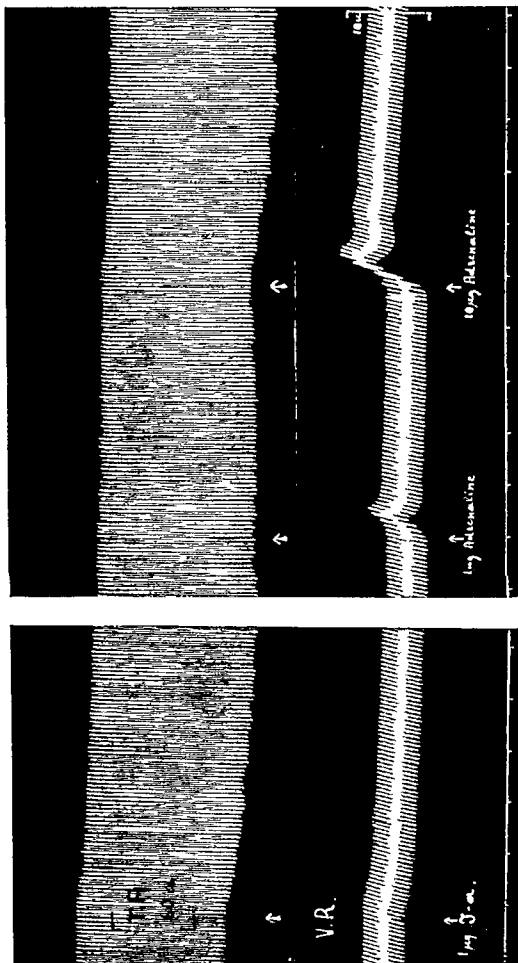


FIG. 2 I P L Dog, 14.9 kgm  
Comparison of the effect of 1 microgm I<sup>a</sup> and of 10 microgm epinephrine on T and V R

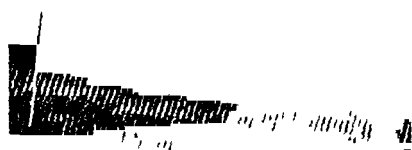
times accompanied by a fall in the venous reservoir volume indicating an increase in capacity of the blood vessels, but this response was not so regularly observed (figs. 1 and 2). These actions were in striking contrast to those of

V.R



P.A.p

- 9 mm blood



A

B

FIG 3. I.P.L. Dog, 12.7 kgm.

At A. and B. 2 microgm. I-a, Cocaine (1 mgm.) and epinephrine (10 microgm.) having been administered between the two tests.

epinephrine which over a wide range of doses (1-100 microgm.) always produced a rise in P.A.p. (figs. 1 and 2) and a fall in lung blood volume (fig. 1). The differences could be observed when equal doses of the two drugs were used (fig. 2) but they were still more marked if the doses were adjusted to have equal

bronchodilator actions (figs 1 and 2) when the amount of adrenaline was five to ten times that of I-a

The pulmonary vasomotor response to I-a was not significantly altered by cocaine (fig 3) or atropine. It may have been intensified by the administration of ergotamine or a related compound when the effect of such administration had been to increase the perfusion pressure much above its original level. This consideration may apply to the experiment illustrated in figs 4a and 4b. In this case I-a was given not by injection into the blood but as an inhalation (spray made of 1 per cent solution). In the first test (fig 4a) the inhalation was continued for four respirations, then, after injection of dihydroergotamine (1 mgm) which raised the perfusion pressure to an unusually high level (from 18.9 to 23.9 cm blood) a second and third test for two and fifteen respirations, respectively, were applied. Since we have not measured the amount of drug which was taken up by the lungs when it was given in this way and since the time of exposure in the three tests varied, the responses obtained cannot be assessed quantitatively. It may be noted, however, that the fall in P.A.p. following the third inhalation was larger than any similar effect observed when I-a was injected into the blood stream, even when the doses were increased to 20-30 times the threshold value. This, we believe, was in part due to the high pressure at the time when the second and third inhalations were given, since we have noticed that the vasodepressor action of I-a is generally larger when the pressure is higher.

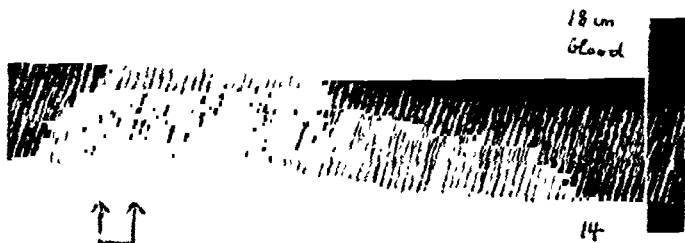
In no case was there evidence that ergotamine or its derivatives could suppress or reverse the vasodilator action of I-a.

*Bronchomotor responses to N-isopropyl norepinephrine.* I-a caused bronchodilatation when given in doses of 1 microgm and upwards (figs 1 and 2). This response was observed both in untreated lungs and in lungs in which bronchoconstriction had already been induced by injection either of histamine or of parasympathetic drugs, including pilocarpine and acetylcholine. The tidal air increases obtained with 1 microgm I-a were between 5 and 10 per cent and persisted for at least five minutes.

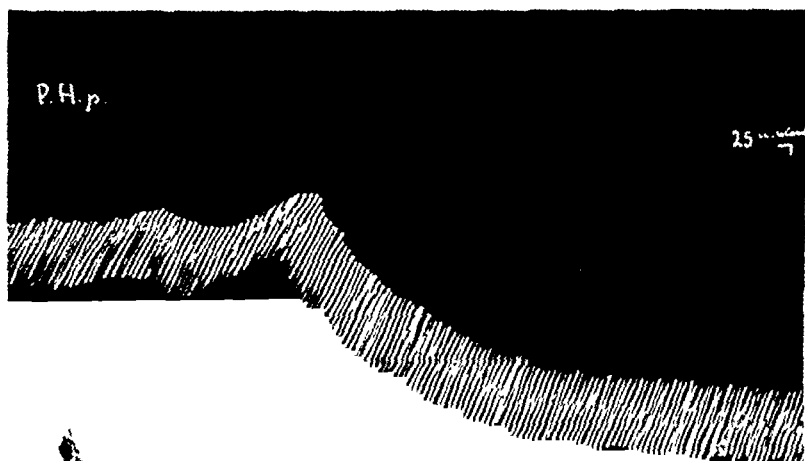
When the bronchodilator actions of I-a and epinephrine were compared during the first one or two hours of perfusion it was found that I-a had the greater activity, to produce an identical response the dose of I-a required was as little as one tenth the dose of epinephrine (fig 2). Rarely, the activity was only five times that of epinephrine. The stronger bronchodilator action of I-a could also be inferred from the fact that preparations which were insensitive to epinephrine (10 microgm) nevertheless responded to I-a (1 microgm, see fig 1). When perfusion was prolonged for more than one to two hours, epinephrine quite frequently produced a biphasic response in which bronchoconstriction, following a brief bronchodilatation, predominated (see also ref 13). Even under these conditions I-a had a powerful bronchodilator action. We should like to emphasize here that I-a unlike epinephrine did not, in our experiments, show any evidence of bronchoconstrictor activity. The bronchodilator effect of I-a was also observed when it was administered as an inhalation (spray made



P.H.p.



a



15



b

FIG. 4. I.P.L. Dog, 16.6 kgm.

a: Inhalation of I-a spray (made from 1 per cent solution) between arrows (four respirations)

b: Two similar tests made after administration of 1 mgm. dihydroergotamine. At the first signal, inhalation for 2 respirations: at the second, inhalation for 15 respirations. Note: time = 10 seconds.

of 1 per cent solution). In one case the expiratory volume (equivalent to tidal air volume) was increased 10 per cent after four successive inhalations.

**DISCUSSION.** The present experiments are in agreement with those of Konzett (2) who found that in the anesthetized dog under positive pressure ventilation *N*-isopropyl-norepinephrine has a bronchodilator action approximately ten times stronger than that of epinephrine. The difference in activity of the two drugs is even greater when allowance is made for the higher molecular weight of I-a. This estimate of the activity of I-a in relation to that of epinephrine more nearly accords with clinical experience of the drug (Dautrebande, 15; Charlier, 16) than do assays made on the perfused isolated guinea pig lungs, from which it has been found that I-a is approximately equal to epinephrine as a bronchodilator agent (Lands *et al.*, 7). This suggests that the dog lungs under either negative or positive pressure ventilation provide a more reliable means for the assay of bronchomotor drugs than do perfused guinea pig lungs. This conclusion is subject to the reservation that the assay carried out on the perfused dog lungs should be made during the first one to two hours of perfusion.

A new fact which emerges from these experiments is that I-a has a pulmonary vasodilator action. This can be shown when the drug is given either by injection into the bloodstream or by inhalation of a spray through the airways. It cannot be assumed, without further evidence, that the drug would also have a dilator action on the lung vessels of the unanesthetized animal or that its total effect would be to reduce the pulmonary arterial pressure since the cardiac output may be simultaneously increased (Konzett, 10). Nevertheless the value of such an action is worth considering. In the first place a diminished resistance in the pulmonary vascular bed will reduce the load on the right heart. A second point relates to the observation that asthmatics may have a low arterial blood oxygen (Verzar and Voegtlin, 17) which in most cases is immediately restored to normal by inhalation of I-a. It is possible that the rapidity with which this occurs is due to an improvement of blood flow through the lungs as well as to the strong bronchodilator effect of the drug. Both actions should improve the conditions for gas exchanges in the alveoli. In this connection it should be mentioned that the beneficial effect of aminophylline, especially in epinephrine-resistant asthmatics, has been attributed to its pulmonary vasodilator action rather than to its relatively weak bronchodilator properties (Lehmann and Young, 18). On the other hand, in unanesthetized dogs aminophylline has been found to increase the pulmonary arterial pressure (Friedberg *et al.*, 19). This does not exclude pulmonary vasodilatation since the rise in P.A.p. may be due to an increased cardiac output which more than offsets a reduced resistance. Nevertheless the question concerning the importance of pulmonary vasodilatation remains unsettled and can only be satisfactorily answered when direct observations of pulmonary arterial pressure, cardiac output, and systemic blood pressure are made on asthmatic patients.

The suggestion has been made that I-a may represent a substance which has only the inhibitory properties of sympathin and lacks its excitatory actions

(Lands *et al.*, 7; Marsh *et al.*, 9; Gilman, 20). Since I-a has a pronounced positive inotropic and positive chronotropic cardiac action and increases the blood sugar (Konzett, 10) we should prefer to regard it as having predominantly inhibitory epinephrine-like actions without being altogether devoid of excitatory properties. This is in agreement with the finding that in the cat the systemic vaso-depressor action of I-a can be reversed by pilocarpine and other parasympathetic substances (Fromherz, 21). Similarly the vasodepressor effect of epinephrine obtained either by giving small doses or by injection after ergotamine can be reversed in the same way by pilocarpine (Konzett, 22). Further, the vaso-depressor actions of I-a and epinephrine can be restored if, following pilocarpine, atropine is also given (Fromherz, 21; Konzett, 22). It would appear then that both I-a and epinephrine are amphotropic substances with inhibitory and excitatory properties. The fact that the direction of the response can be varied might be attributed either to changes in the sensitivity of any given group of receptor cells or to unmasking of other receptor cells.

#### SUMMARY

1) In isolated, blood-perfused dog lungs, with either negative or positive pressure ventilation, N-isopropyl-norepinephrine causes pulmonary vasodilatation. Such responses are obtained when the drug is administered by intravascular injection or by inhalation of a spray.

2) Cocaine, atropine, pilocarpine or ergotoxine (and related compounds) do not alter the direction of the response.

3) Under all of the conditions of experimentation employed here, N-isopropyl-norepinephrine has a strong bronchodilator action, its activity usually being ten times greater than that of epinephrine.

ACKNOWLEDGEMENTS. Part of the expenses of this research were defrayed by a grant to one of us (C. O. H.) from the Earl of Moray Endowment Fund.

#### REFERENCES

1. KONZETT, H.: *Klin. Wschr.*, 51: 1303, 1940.
2. KONZETT, H.: *Arch. f. exper. Path. u. Pharmacol.*, 197: 27, 1940.
3. KONZETT, H., AND RÜSSLER, R.: *Arch. f. exper. Path. u. Pharmacol.*, 195: 71, 1940.
4. FROMHERZ, K.: *Experientia*, 2: 146, 1946.
5. LISSAK, K., MARTIN, J., AND ANGYAN, A.: *Orvosok Lapja.*, 2: 129, 1946.
6. HAMBURGER, J., MILLIEZ, P., AND HALPERN, B.: *Bull. mem. Soc. méd. hôp. de Paris.*, 62: 600, 1946.
7. LANDS, A. M., NASH, V. L., MCCARTHY, H. M., GRANGER, H. R., AND DERTINGER, B. L.: *THIS JOURNAL*, 90: 110, 1947.
8. SIEGMUND, O. H., GRANGER, H. R., AND LANDS, A. M.: *THIS JOURNAL*, 90: 254, 1947.
9. MARSH, D. F., PELLETIER, M. M., AND ROSS, C. A.: *THIS JOURNAL*, 92: 108, 1948.
10. KONZETT, H.: *Arch. f. exper. Path. u. Pharmacol.*, 197: 41, 1940.
11. DOMINI, G.: *Boll. Soc. Ital. Biol. Sper.*, 18: 710, 1943.
12. DALY, I. DE B.: *Quart. J. exper. Physiol.*, 28: 357, 1938.
13. DALY, I. DE B., HEBB, C. O., AND PETROVSKAIA, B.: *Quart. J. exper. Physiol.*, 31: 129, 1941.
14. HEBB, C. O., AND NIMMO SMITH, R. H.: *J. Physiol.*, 105: 1, 1946.

- 15 DAUTREBANDE, L Aerosols medicamenteaux Gembloux Belgium, J Ducolot Paris, Masson, 1946
- 16 CHARLIER, R Med Times, 75 277, 1947
- 17 VERZAR, F, AND VOFGLIN, W Schweiz med Wschr, 77 980, 1947
- 18 LEHMANN, G, AND YOUNG, J W THIS JOURNAL, 83 90, 1943
- 19 FRIEDBERG, L, KATZ, L N, AND STAINITZ, F S THIS JOURNAL, 77 80, 1943
- 20 GILMAN, A J Allergy, 19 281, 1943
- 21 FROMHERZ, K Festschr f E Chr Barell p 327, Basel, 1946
- 22 KOVZETT, H Experientia, 4 403, 1948

# TUBERCULOCIDAL ACTIVITY AND TOXICITY OF SOME DIPHENYLMETHANE DERIVATIVES

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The germicidal and fungicidal properties of diphenylmethane derivatives have been rather extensively studied (1-13). A search of the literature has failed to reveal, however, any investigation of the possible antibacterial effects of such compounds against tubercle bacilli.

During routine screening of compounds for antibacterial activity, a number of diphenylmethane derivatives were studied, certain of which have indicated extremely high inhibitory action against *Mycobacterium tuberculosis*. These compounds appeared particularly interesting upon observation that their action was mainly bactericidal in nature. Of further interest was the relation exhibited between chemical structure and antibacterial property.

**EXPERIMENTAL.** *In vitro study.* Data reported here were obtained with the No. 607 avirulent strain of *M. tuberculosis*. All tests were carried out in Proskauer and Beck synthetic broth (pH 7.0), which was prepared in accordance with the modifications suggested by Youmans (14).

A 4-day old culture of tubercle bacilli grown on Proskauer and Beck agar at 37°C. served as the source of inoculum, 0.1 mgm. of growth (moist weight) being inoculated onto the surface of 10 cc. of broth in 25 x 150 cm. test tubes. End-points were determined after three, five and seven days incubation. Where results permitted, two readings were made: (1) the concentration showing complete inhibition of growth, and (2) that concentration at which 50 per cent of control growth was present. Compounds showing complete inhibition were tested for bactericidal properties by subculturing into 10 cc. of plain Proskauer and Beck broth, inoculating the largest amount of drug-broth possible (0.1 to 1.0 cc.) without introducing an inhibitory concentration *per se*. All subcultures were incubated at 37°C. for seventeen days.

Because of the low solubility of most of the derivatives, stock solutions were prepared by dissolving the respective compounds in 95 per cent ethyl alcohol to a concentration of 500 mgm. per cent. Subsequent dilutions were made aseptically in the test broth so that each compound was tested at a maximum of 4.0 mgm. per cent, with lower concentrations decreasing by halves. While controls on the diluent were included with each run, it was found in repeated tests that the concentration of ethyl alcohol in broth necessary to inhibit completely the test strain of *M. tuberculosis* was eight times that present in the initial tube of the dilution series.

*Toxicity study.* Acute toxicities were determined in white mice (CFW) following oral administration of the compounds. Animals weighing 20 grams each were used throughout, and the respective derivative was suspended in 10 per cent gum acacia in concentration yielding the required dose in a volume of 0.5 cc. Food was withheld for sixteen hours prior to dosing and for eight hours after. Water was freely allowed. Mice were observed for a period of seven days following dosage. Because of lack of an adequate method for the determination of diphenylmethane derivatives in body fluids, absorption studies were not attempted at this time.

**RESULTS.** Data obtained with diphenylmethane and 28 derivatives are summarized in table 1. Structure of the compounds has been included to facilitate correlation of chemical constitution with antibacterial activity and toxicity. All compounds effecting complete inhibition of growth proved bactericidal at the respective concentrations indicated.


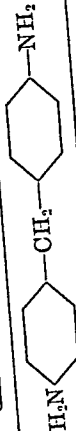
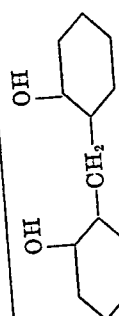
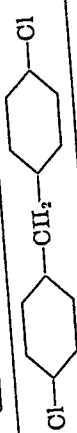
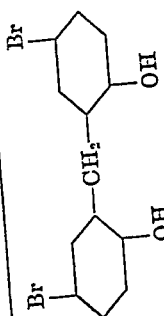
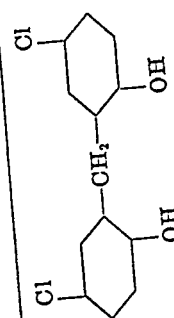
The parent compound (No. 1) showed no activity at 4.0 mgm. per cent. Substitution of amino groups (No. 2) or chlorine atoms (No. 4) at the para positions, or hydroxy groups (No. 3) ortho to the methylene linkage produced only slight change in the degree of activity. On the other hand, substitution of either bromine (No. 5) or chlorine (No. 6) in the 5,5'-positions, with hydroxy groups occupying the 2,2'-positions in each case, resulted in greatly increased antibacterial property; both derivatives inhibited growth completely at a concentration of 0.25 mgm. per cent. A decided decrease in activity was apparent, however, upon the addition of a second hydroxy group to the ring (No. 9). Although substitution of an additional pair of chlorine atoms at the 3,3'-positions (No. 7) showed no advantage over the dichloro derivative (No. 6), the 3,3',5,5',6,6'-hexachloro derivative (No. 8) manifested a four fold enhanced bactericidal activity. Replacement of the 3,3'-chlorine atoms in No. 7 with iodine yielded a compound (No. 13) with slightly better antibacterial activity. Introduction of methyl groups (No. 10) in the 4,4'-positions of No. 6 produced a significant decrease in inhibitory action, as did the addition of a hydroxymethyl group in the 3-position (No. 11). A still further decrease in activity resulted with the 3,3'-bis(hydroxymethyl) derivative (No. 12). With the exception of No. 21, all other derivatives containing both methyl and propyl substituents (Nos. 19, 22, 23, and 24) were comparatively devoid of activity. Marked reduction in inhibitory action was also apparent as the result of replacement of the 5,5'-chlorine atoms with nitro (No. 16), arsono (No. 17), *tert.*-butyl (No. 18), or *tert.*-amyl groups (No. 20). Substitution of a methyl group (No. 27) or phenyl group (No. 29) on the methylene linkage had little effect on activity. While chlorine in the 4,4'-positions (No. 4) of the parent compound produced some increase in activity, further substitution of a trichloromethyl group on the methylene linkage (No. 28) appeared to nullify this effect, 4.0 mgm. per cent showing no inhibition whatever.

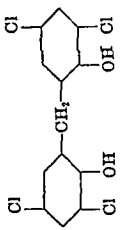
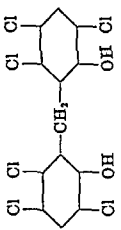
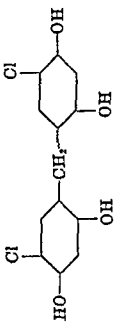
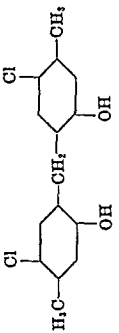
Although acute toxicity tests were performed on only the most active of the derivatives, the results obtained offer sufficient information to justify several general conclusions. Considering single halogen substituents on the ring, bromine appeared less toxic than chlorine, No. 5 having an LD<sub>50</sub> of 2.0 gm./kgm., while 100 per cent mortality resulted with No. 6 at 1.5 gm./kgm. Both compounds, however, possessed equal antibacterial activity. Replacement of the halogens with a nitro group (No. 16) resulted in considerable decrease in toxicity, LD<sub>50</sub> greater than 4.0 gm./kgm., although activity was likewise markedly reduced. Increasing the number of chlorine atoms on the ring was attended by an increase in toxicity, the LD<sub>50</sub> of No. 7 being 0.5 gm./kgm., with No. 8 producing 50 per cent deaths at 0.08 gm./kgm. In this respect, No. 8 while being the most toxic was also the most active of the compounds against tubercle bacilli.

TABLE 1  
Activity in vitro against tubercle bacilli (#607) and toxicity of diphenylmethane derivatives

TABLE 1

Activity *in vitro* against tubercle bacilli (#607) and toxicity of diphenylmethane derivatives

compound*			ACTIVITY IN NGM. PER CENT AFTER 7 DAYS INCUBATION		ACUTE ORAL TOXICITY IN MICE
No.	Name	Structure	100% Inhibition†	50% Inhibition	LD <sub>50</sub> gm./kgm.
			>4.0	>4.0	
1	Diphenylmethane		>4.0	>4.0	
2	4,4'-Diaminodiphenyl- methane		>4.0	4.0	
3	2,2'-Dihydroxydiphenyl- methane		>4.0	2.0 -4.0	
4	4,4'-Dichlorodiphenyl- methane		>4.0	0.5 -1.0	2.0
5	Bis(2-hydroxy-5-bromo- phenyl)methane		0.25	0.125-0.25	
6	Bis(2-hydroxy-5-chloro- phenyl)methane		0.25	0.125-0.25	1.0

7	Bis(2 hydroxy 3,5 di-chlorophenyl)methane		0 5	0 25-0 5	0 5
8	Bis(2 hydroxy 3,5,6 tri chlorophenyl)methane		0 063	0 016-0 031	0 08
9	Bis(2,4 dihydroxy 5 chlorophenyl)methane		2 0	1 0 -2 0	2 0
10	Bis(2 hydroxy 4 methyl 5 chlorophenyl)methane		1 0	0 5	2 5

\* Compounds Nos 1 and 2 were obtained from Eastman Kodak Co, Nos 6 and 8 from Givaudan Delawanna, Inc, No 15 from Heyden Chemical Corp, No 28 from Merck & Co, Inc. All other compounds were synthesized in this laboratory by H E Faith, details of synthesis will be described elsewhere (33)

† Concentrations = bactericidal

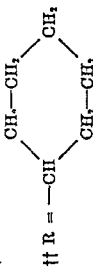




TABLE 1—Continued

No.	Name	Structure	ACTIVITY IN MGK. PER CENT AFTER 7 DAYS INCUBATION		ACUTE ORAL TOXICITY IN MICE
			100% Inhibition†	50% Inhibition	
11	2,2'-Dihydroxy-3-hydroxymethyl-5,5'-dichlorodiphenylmethane		2.0	1.0	LD <sub>50</sub> gm./kgm. >4.0
12	Bis(2-hydroxy-3-hydroxymethyl-5-chlorophenyl)methane		>4.0	>4.0	
13	Bis(2-hydroxy-3-iodo-5-chlorophenyl)methane		0.25	0.125	1.0
14	2,2',6',2''-Dimethylenetris(4-chlorophenol)		>4.0	>4.0	

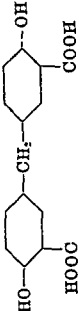
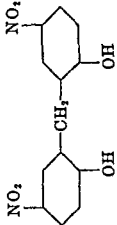
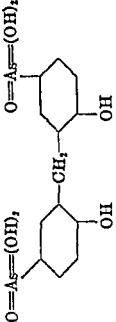
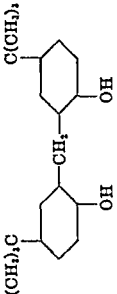
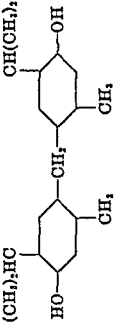
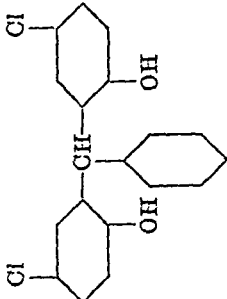
15	Bis(3-carboxy-4-hydroxyphenyl)methane		>4.0	>4.0	>4.0
16	Bis(2-hydroxy-5-nitrophenyl)methane		4.0	1.0-2.0	>4.0
17	Bis(2-hydroxy-5-arsono-phenyl)methane		>4.0	>4.0	>4.0
18	Bis(2-hydroxy-5-tert-butylphenyl)methane		>4.0	4.0	>4.0
19	Bis(2-methyl-4-hydroxy-5-isopropylphenyl)methane		>4.0	>4.0	>4.0

TABLE 1—Continued

No.	Name	Structure	ACTIVITY IN MG. PER CENT AFTER 7 DAYS INCUBATION		ACUTE ORAL TOXICITY IN MICE
			100% Inhibition†	50% Inhibition	
			>4.0	>4.0	LD <sub>50</sub> gm./kgm.
20	Bis(2-hydroxy-5- <i>tert</i> - amylphenyl)methane		>4.0	>4.0	
21	2,2'-Dihydroxy-3-iso- propyl-5,5'-dichloro-6- methyl-diphenylmeth- ane		0.125	0.063-0.125	4.0
22	2,2'-Dihydroxy-3-isopro- pyl-5-chloro-5'- <i>tert</i> - butyl-6-methyldiphen- ylmethane		>4.0	>4.0	
23	Bis(2-hydroxy-3-isopro- pyl-5-chloro-6-methyl- phenyl)methane		>4.0	>4.0	

24	2,4'-Dihydroxy-6,2'-dimethyl-3,5'-diisopropyl-5-chlorodiphenylmethane		>4.0	>4.0	>4.0	0.75
25	Bis-dicyclohexylamine salt of bis(2-hydroxy-5-chlorophenyl)methane		0.5	1.0	>4.0	2.0
26	1,1'-Bis(2-hydroxynaphthyl)methane		>4.0	>4.0	>4.0	2.0
27	Methyl-bis(2-hydroxy-5-chlorophenyl)methane		0.25-0.5	0.5	>4.0	2.0
28	1,1-Di-4-chlorodiphenyl-2,2,2-trichloroethane		>4.0	>4.0	>4.0	2.0

TABLE 1—Continued

No.	Name	Structure	ACTIVITY IN MG. PER CENT AFTER 7 DAYS INCUBATION		ACUTE ORAL TOXICITY IN MICE
			100% Inhibition†	50% Inhibition	
29	Phenyl-bis(2-hydroxy-5-chlorophenyl)methane		0.25	0.063-0.125	LD <sub>50</sub> gm./kgm. 3.0

The toxic effects manifested by the presence of a single chlorine atom and hydroxy group on each ring (No. 6) were significantly reduced by the addition of a second hydroxy group (No. 9), a methyl group (No. 10), or a hydroxymethyl group (No. 11). Decrease in toxicity, nevertheless, was again accompanied by a decrease in antibacterial activity. Addition of iodine (No. 13), on the other hand, did not appreciably alter toxicity or activity.

Replacement of a hydrogen atom on the methylene linkage with a methyl group (No. 27) or phenyl group (No. 29) resulted in greatly reduced toxicity, whereas activity against tubercle bacilli remained practically unchanged (compare No. 6). Inclusion of the 3-isopropyl and 6-methyl groups yielded a compound (No. 21) with much reduced toxicity, LD<sub>50</sub> of 4.0 gm./kgm., and slightly better inhibitory action than No. 6. While strong activity was maintained by conversion of No. 6 to its dicyclohexylamine salt (No. 25), this derivative did not prove any less toxic.

**Discussion.** Investigation of the present series of diphenylmethane derivatives has indicated that activity of the parent compound against tubercle bacilli is essentially increased by nuclear halogen substitution. Further enhancement of this activity resulted when both halogens and hydroxy groups were present. A particular feature of No. 21 seems worthy of mention in view of recent trends in tuberculosis therapy, namely, that a major portion of the molecule consists of chlorothymol. Caujolle, Franck and Heynard (15) reported that methyl, ethyl, propyl, isopropyl, butyl, isobutyl and benzyl ethers of thymol had a predilection for lung tissue following intravenous administration to dogs. According to Perlman, Brown and Raiziss (16) the subcutaneous administration of thymol to guinea pigs infected with a virulent bovine strain of *M. tuberculosis* rendered the infection milder and increased longevity; in many cases it was impossible to recover tubercle bacilli from the various organs. McBurney and co-workers (17, 18) studied the effect of oral administration of thymol in both experimentally induced tuberculosis and actual clinical cases. They concluded that this type of medication, especially in early recognized human pulmonary forms, was worthy of further investigation.

In addition to its marked activity against tubercle bacilli, the iodine compound (No. 13) offers interesting possibilities for future study of iodo-derivatives, particularly in regard to the role reportedly played by iodine itself in the metabolism of the tubercle bacillus. Jobling and Petersen (19, 20) found that iodine could unite with the unsaturated fatty acids obtained from this organism, bringing about neutralization of their "ferment-inhibiting" properties. This action of iodine was also exerted on the ferment-inhibiting substances present in caseous matter. Emphasizing the importance of their observations in the search for an effective drug against tuberculosis, they believed that iodine may thus serve to facilitate solution and absorption of the caseous material, thereby exposing the bacilli to the action of the therapeutic agent. While a number of attempts to treat tuberculosis with iodine and iodine compounds have not been too encouraging (21-29), it has lately been shown by Woody and Avery (30) that the therapeutic effect of streptomycin against established tuberculosis in guinea

pigs could be significantly enhanced by the simultaneous administration of potassium iodide. Both *in vitro* and *in vivo* tests are now in progress to determine the actual value of these findings, and to ascertain whether iodine in the form existing in No. 13 may have any contributing effect in therapy of experimental tuberculosis. Investigation of the efficacy *in vivo* of other members of this series of compounds is likewise being made, the results of which will be reported elsewhere (31).

Thirteen of the 29 compounds exhibited bactericidal action against tubercle bacilli at concentrations of 4.0 mgm. per cent and less. It might be of interest to mention that when tested with various species of bacteria, the diphenylmethane derivatives showed much greater activity against Gram-positive organisms than against Gram-negatives (32). Whereas 26 of the compounds were bactericidal for the former type, only six were effective against Gram-negative species.

#### SUMMARY

1. Twenty-eight derivatives of diphenylmethane have been tested for antibacterial activity against tubercle bacilli. Thirteen of the compounds were bactericidal at concentrations ranging from 0.063 to 4.0 mgm. per cent.

2. An attempt has been made to correlate both activity and toxicity with chemical constitution.

3. Activity, in general, appeared dependent upon halogen and hydroxy group substitution in the parent structure, and more particularly upon position.

4. Bis(2-hydroxy-5-bromophenyl)methane completely inhibited growth of tubercle bacilli at 0.25 mgm. per cent, with an  $LD_{50}$  for mice of 2.0 gm./kgm.

The corresponding dichloro derivative was equally as active but twice as toxic. Compounds with other radicals in the 2,2'-5,5'-positions possessed only slight or no demonstrable inhibitory action, namely, 2,2'-dihydroxy-5,5'-dinitro; 2,2'-dihydroxy-5,5'-diarsono; 2,2'-dihydroxy-5,5'-di-*tert.*-butyl; 2,2'-dihydroxy-5,5'-di-*tert.*-amyl; and 2,2'-dimethyl-5,5'-di-isopropyl.

5. Bis(2-hydroxy-3,5,6-trichlorophenyl)methane was the most active of the derivatives studied, effecting complete inhibition at 0.063 mgm. per cent. It was also, however, the most toxic,  $LD_{50}$  of 0.08 gm./kgm.

#### REFERENCES

1. KLARMANN, E., AND VON WOWER, J.: J. Am. Chem. Soc., **51**: 605, 1929.
2. KLARMANN, E., GATES, L. W., AND SHTERNOV, V. A.: J. Am. Chem. Soc., **54**: 3315, 1932.
3. HARDEN, W. C., AND REID, E. E.: J. Am. Chem. Soc., **54**: 4325, 1932.
4. SUTER, C. M.: Chem. Rev., **28**: 269, 1941.
5. GUMP, W. S.: U. S. Patent 2,250,480, 1941.
6. TRAUB, E. F., NEWHALL, C. A., AND FULLER, J. R.: Surg. Gynec. and Obst., **79**: 205, 1944.
7. GUMP, W. S.: Soap Sanit. Chemicals, **21**: 36, 50, 85, 1945.
8. FISHER, E., AND LÓPEZ, M. A.: Bol. soc. biol. Santiago, Chile, **2**: 376, 1945.
9. FISHER, E., GARCÉS, C., AND LÓPEZ, A.: Science, **102**: 507, 1945.
10. MARSH, P. B., AND BUTLER, M. L.: Indust. and Eng. Chem., **38**: 701, 1946.
11. FISHER, E., GARCÉS, C., AND LÓPEZ, A.: J. Bact., **51**: 1, 1946.
12. FULLER, J. R., NEWHALL, C. A., THORNE, F. C., AND TRAUB, E. F.: Am. J. Pub. Hlth., **38**: 1228, 1948.

13. BERK, S.: *Indust. and Eng. Chem.*, **40**: 262, 1948.
14. YOUNG, G. P.: *Proc. Soc. Exper. Biol. and Med.*, **57**: 122, 1944.
15. CAUJOLLE, F., FRANCK, C., AND HEYNARD, J.: *Bull. Acad. Nat. Med.*, **131**: 94, 1947.
16. PERLMAN, H. H., BROWN, H., AND RAIZISS, G. W.: *Am. Rev. Tuberc.*, **44**: 83, 1941.
17. MCBURNEY, R., CASON, L., AND SEARCY, H. B.: *J. Lab. and Clin. Med.*, **30**: 32, 1945.
18. SEARCY, H. B., MCBURNEY, R., AND ROWE, H. S.: *J. M. A. Alabama*, **11**: 217, 1942.
19. JOBLING, J. W., AND PETERSEN, W.: *J. Exper. Med.*, **19**: 251, 1914.
20. JOBLING, J. W., AND PETERSEN, W.: *J. Exper. Med.*, **19**: 383, 1914.
21. TURMANN, J.: *Münch. med. Wochschr.*, **56**: 1532, 1909.
22. PERTIK, T.: *Deut. med. Wochschr.*, **39**: 75, 1913.
23. NORDELLI, G.: *Arch. farm. sper.*, **28**: 41, 1919.
24. BONNA-MOUR, AND DELORE, P.: *Compt. rend. soc. de biol.*, **95**: 467, 1926.
25. KNATSI, G.: *J. Infect. Dis.*, **50**: 261, 1932.
26. KOLMER, J. A., AND BROWN, H.: *J. Chemotherapy*, **10**: 73, 1934.
27. SMITH, L. W.: *J. Lancet*, **58**: 195, 1938.
28. SAZ, A. K., JOHNSON, F. R., BURGER, A., AND BERNHEIM, F.: *Am. Rev. Tuberc.*, **48**: 40, 1943.
29. FREEDLANDER, B. L., AND FRENCH, F.: *Proc. Soc. Exper. Biol. and Med.*, **63**: 319, 1946.
30. WOODY, E., JR., AND AVERY, R. C.: *Science*, **108**: 501, 1948.
31. COLE, V. V., AND HULPIEU, H. R.: to be published.
32. BAHLER, M. E., AND FLORESTANO, H. J.: *J. Bact.*, to be submitted.
33. FAITH, H. E.: *J. Am. Chem. Soc.*, to be submitted.



# THE FATE OF TWO ARSENOXIDES IN THE DOG

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The measurement of elemental arsenic in biological material is not a measure of the potential therapeutic activity of a phenylarsenoxide. It has been known since Ehrlich that the high parasitocidal activity of organic arsenicals depends on the bond between aromatic carbon and metal, as well as on the nature of side chains on the aromatic ring. Thus, although Eagle (1) was able to show that arsenic as oxophenarsine hydrochloride was antispirochetal at dilution of 1:3,000,000 Magnuson and Raulston (2) are careful to point out that the finding of arsenic in tissues and particularly in plasma at this concentration, after administration of oxophenarsine, does not indicate that a true spirocheticidal level is present since the active component is not really being measured.

Several attempts have been made to fill this gap. Using chemical and biological methods of assay in parallel, both Hawking (3) and Wright and Peters (4) found no correlation between the arsenic level in body fluids and the trypanocidal titer of these fluids. Hawking worked chiefly with the pentavalent tryparsamide, but Wright and Peters used the trivalent oxophenarsine hydrochloride. The biological approach has the disadvantage of being only applicable to the organism studied, i.e., the above results would not necessarily apply to experimental or human syphilis. Secondly, these methods are entirely nonspecific, and tell nothing of chemical disposition of the drugs used.

An earlier series of attempts to study the fate of arsenic in the body centered about arspenamine. In 1922, two papers appeared describing a reaction for arspenamine in urine (5, 6). In the following year, Kolls and Youmans (7) extended these methods to the direct determination of the o-aminophenol moiety of arspenamine in blood, organs and urine. Their chemical methods were the starting point of the present study.

This report deals with oxophenarsine hydrochloride (m-amino-p-hydroxyphenylarsenoxide, HCl salt) and m-amino-p-carbamylphenylarsenoxide in the dog, by performing parallel determinations of total arsenic and aromatic amine on the same samples of plasma and urine, collected at varying intervals after the intravenous administration of drug. It seemed reasonable to assume that if the two determinations agreed in terms of the original molecule, the drug was present as such. (The alternative hypothesis, that the drug is split to an arsenic and to an amino moiety which circulate and are excreted in equivalent amounts, appears remote.) However, oxidation or reduction of the arsenic, yielding the corresponding arsonic acid or arsenobenzene, cannot be ruled out, since in either of these cases the arsenic-amino relationship would be that of the original arsen-

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oxide. As with all colorimetric methods, the data here cannot be taken as ultimate proof of the identity of the compounds. Only their isolation would permit such a conclusion. The present evidence is therefore regarded as suggestive rather than final.

**CHEMICAL METHODS.** *Oxophenarsine Hydrochloride: Determination of o-aminophenol moiety.* Diazotized ortho-aminophenols will not couple with amines to give a highly colored azo dye; thus, the Bratton and Marshall (8) procedure or modifications of it could not be used. As indicated above, the work of Kolls and Youmans on arspenamine was applicable to the present problem, since the o-aminophenol group is common to arspenamine and oxophenarsine. To obtain greater accuracy and particularly to eliminate a "blank", radical changes were made. The principle of the method is coupling of diazotized o-aminophenols in alkaline solution with 1,3 dihydroxyphenols to yield a yellow-red dye. Of a large number of potential coupling agents tried, hexylresorcinol (1,3 dihydroxy-4-hexylbenzene) was found to be the best from a standpoint of high color intensity and low blank value. Both of these factors are also dependent on the final pH, which is 9.8 in this procedure.

This method does not determine aryl amino compounds as such, i.e., sulfanilamide and m-aminophenylarsenoxide do not give the color. All o-aminophenols tested, however, do give the reaction. This includes the parent o-aminophenol, m-amino-p-hydroxyphenylarsonic acid and m-amino-p-hydroxybenzoic acid, methyl ester (orthoform).

There is apparently an unknown substance in dog plasma and urine which enhances the absolute value of the color obtained in this reaction. This substance appears to react with the diazonium compounds of o-aminophenols (including oxophenarsine hydrochloride) in acid solution to form a new complex which gives a deeper color with hexylresorcinol than do the diazo salts of o-aminophenols. Thus, the "absolute value" of the optical density of the final solution (known amount of o-aminophenol or oxophenarsine minus blank) is approximately 50 per cent higher for plasma than it is for aqueous solutions or dilute (1:100) urine. For plasma, this enhancement factor has been found to be constant, even from dog to dog. The blank was also quite constant, varying in optical density under these conditions between .118-.127, which is a variation of less than 0.5 micrograms. Nevertheless, a standard curve was always prepared from the plasma of each dog before each experiment. Under the conditions described below, 10 micrograms of oxophenarsine hydrochloride added to plasma had an "absolute" optical density value of .200 (.325-.125), whereas this amount added to aqueous solutions or to dilute urine gave a value of .130 (.185-.055).

This enhancement was observed when oxophenarsine was added either to plasma, or to filtrate of plasma prepared by trichloroacetic acid precipitation.

Recovery experiments were done by comparing the values obtained in two types of experiments. In the first type, which simulated the conditions of actual practice, known amounts of oxophenarsine hydrochloride were added to plasma. In the second type, the drug was added to the plasma filtrate. The difference in the values obtained represented loss of drug in the precipitation of

protein. This did not exceed 15 per cent, and replicate determinations of the first type gave, on the same plasma, agreement within 5 per cent.

*Procedure for Plasma.* Five cc. of plasma were added to 20 cc. of distilled water. Five cc. of 15 per cent trichloroacetic acid were added and the precipitate filtered through No. 12 Whatman paper. To 20 cc. of the filtrate, 1 cc. of 0.1 per cent aqueous  $\text{NaNO}_2$  was added. After 3 minutes, 10 cc. of 15 per cent  $\text{Na}_2\text{CO}_3$ , and 1 cc. of 0.1 per cent hexylresorcinol in 50 per cent ethanol were added. After 20 minutes the optical density was read in the Beckmann spectrophotometer, at 500 millimicrons with water as the standard. Barrel-shaped cuvettes, 100 mm. long, were used throughout. Optical density was converted into concentration of oxophenarsine hydrochloride by reference to the standard curve constructed for the plasma of the same dog, using this identical procedure.

*Procedure for Urine.* The procedure for urine is complicated by the fact that unlike its constant concentration in plasma, the "enhancement factor" in urine is variable. Two measures were used, one in the first three experiments reported, the other in the fourth experiment, to minimize this variable. In the first three experiments urine flow and samples for the appropriate collection periods were taken on the day preceding the experiment. Standard curves for each of these periods were run. It was found that the degree of enhancement was inversely related to the amount of urine flow, i.e., as the urine became more concentrated, the concentration of interfering substance increased regularly. Thus for a particular dog and a particular urine flow, a standard was available. The blank, which also varied with urine flow, was of course a part of this standard. The second method is preferable, since the standardization is done directly on the urine sample to be analyzed. In this procedure the "blank" was obtained by running the reaction on the appropriately diluted urine, omitting the hexylresorcinol. A slight correction may be added for the tinctorial value of the hexylresorcinol, as determined separately. The slope of the standardization curve was obtained by running two additional reactions in which 5 micrograms, and 10 micrograms of oxophenarsine were added to the diluted urine and optical density values were obtained for these two points. The O.D. difference between the points gave the 5 microgram value, and since there is a linear relation between concentration and O.D., the slope of the curve was thus obtained. The diluted urine was then run, and the concentration of oxophenarsine present was determined by reference to this curve. In practice, all four of these ("blank", 5 and 10 micrograms, and unknown) were run simultaneously, in duplicate.

The chemical procedure for urine is identical to that for plasma. Urine was diluted 200-1000-fold, and 20 cc. of this solution was added to 5 cc. of 15 per cent trichloroacetic acid. Succeeding steps are given above as for plasma.

*m-Amino-p-Carbamylphenylarsenozide: Determination of arylamine group. Procedure for plasma.* This compound can be determined by the method of Bratton and Marshall (8). A few minor changes were made. Four cc. of plasma were used, and 25 cc. of filtrate were taken for the reaction. Optical density was read 30 minutes after coupling, in 100 mm. cells in the Beckman spectrophotometer at 550 millimicrons. Optical density of the blank averaged .095, and that of 10 micrograms averaged .245.

*Procedure for Urine.* It had been observed previously (9) that there is a substance in dog urine which gives a blank in the Bratton-Marshall method. In sulfonamide studies this was not important since high dilution of urine (as 0.1-1 cc. of 1:100 urine) contained measurable amounts of sulfonamide in most experiments, and at this dilution the interference is generally negligible. However, in the present work much smaller doses of drug were given and a smaller percentage of these excreted. Larger amounts of urine had to be used in the present work to obtain measurable drug. Therefore, it was necessary to use the blank value, to which amounts of the drug were found to be directly additive. The blank value varied with concentration of urine, therefore the rate of urine flow was studied for each dog before the experiment began. Blank values corresponding to known urine flow

were recorded and used as a baseline for the actual determination. The difference between known values and the blank was constant, no matter what the urine flow.

Twenty cc. of urine diluted 200-1000-fold were added to 5 cc. of 15 per cent trichloroacetic acid, and the color developed by the Bratton and Marshall procedure. The final color value was interpreted in terms of the blank found for the particular urine flow involved.

*Method for Total Arsenic.* A modification (10) of the method of Magnuson and Watson (11) was used. The color obtained in the final solution (made up to 30 cc.) was measured in 100 mm. cells in the Beckman spectrophotometer, at 840 millimicrons. Blanks for plasma and urine had an optical density of .030-.060. Ten micrograms of oxophenarsine hydrochloride (3 micrograms of arsenic) gave an optical density of .310 while 10 micrograms of m-amino-p-carbamylphenylarsenoxide (3.3 micrograms of arsenic) gave an optical density of .350.

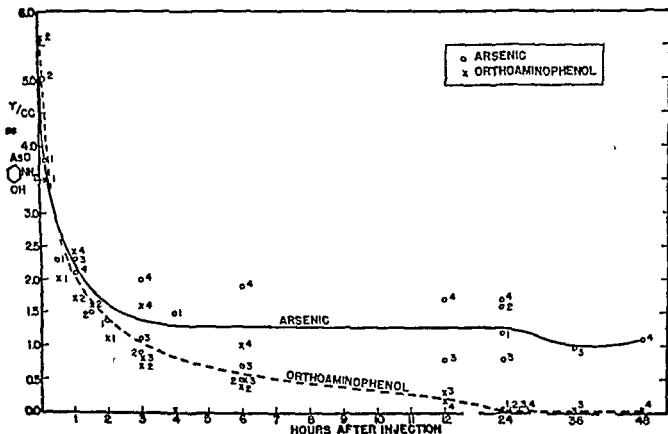


FIG. 1. Plasma concentrations of orthoaminophenol and arsenic moieties following intravenous administration (100 mgm./kgm.) to dogs. The figure at each point represents the average of the four experiments. The lines represent rough averages from the four experiments. These experiments are given in table 1.

*Sensitivity of Analytical Methods.* The lower limit of all these methods is approximately one microgram in the final solution. Since 5 cc. of plasma were used, it was possible to determine as little as 0.2 microgram drug per cc. plasma. For the diazo methods applied to urine the limits depend somewhat, as indicated above, on the concentration of the urine. One to two micrograms could be detected in 20 cc. of urine of dilution varying from 1:200 to 1:1000, depending on the urine flow. For total arsenic in urine, only the size of the sample determines the lower limit of sensitivity.

In all but a few cases, duplicate determinations were done. Results that did not check within 10 per cent were repeated, or in one or two cases where there was insufficient plasma to repeat, the reading was discarded.

**BIOLOGICAL METHODS, AND DRUGS.** Adult female dogs were used. Thirty to 40 cc. of blood were withdrawn into potassium oxalate at each period. The blood was immediately centrifuged at about 2000 R.P.M. for 30 minutes. Plasma was then drawn off and duplicate determinations for the arylamino or the orthoaminophenol group were done at once. The remainder of the sample was set aside for arsenic determinations.

Urine was collected by catheter, after which the bladder was washed with 10 cc. water. Food and water were withheld during the experiment.

Commercial oxophenarsine hydrochloride (Parke, Davis-Mapharsen) was used, directly from the ampule, dissolved in distilled water. The m-amino-p-carbamylphenylarsenoxide was kindly supplied by Dr. G. O. Doak. It was dissolved in a small excess of dilute sodium hydroxide. Both drugs were administered intravenously.

**RESULTS.** *Oxophenarsine Hydrochloride.* Figure 1 shows concentration of drug in plasma following injection of oxophenarsine into four dogs, as estimated by the o-aminophenol and total arsenic reactions. It will be noticed that up to three hours the two sets of values are in close agreement. From three to twelve hours, a marked divergence is apparent; the o-aminophenol moiety is rapidly disappearing whereas arsenic remains relatively constant. After twelve hours, the o-aminophenol constituent has entirely disappeared (less than 0.2 micrograms/cc. as oxophenarsine) but the arsenic level is maintained at about 1 microgram per cc. for at least 48 hours.

Table 1 shows the cumulative urinary excretion for these experiments. The dissociation of o-aminophenol from the arsenic is indicated by the increase in the ratio of OAP/As in the urine. Taking all values, this is 1.3 for the first 3 hours, 1.7 for the next 6 hours, and 1.7 for the 12-48 hour period. Thus, in agreement with its rapid disappearance from plasma, more o-aminophenol than arsenic appears in the urine, and this discrepancy is particularly striking after the third hour.

*m-Amino-p-carbamylphenylarsenoxide.* Figure 2 shows that concentration of arsenic and arylamino group agree closely up to 12 hours in one case and 24 hours in the other. Table 2 shows that these constituents also closely parallel each other in urine. The arylamino/arsenic ratio never deviated from 1 by more than 12 per cent, which is the order of magnitude of analytical error. In striking contrast to oxophenarsine, therefore, this compound appears to circulate as such for 12-24 hours. The possibility of oxidation to the corresponding arsonic acid, however, has not been excluded.

**DISCUSSION.** These results suggest that after intravenous injection of oxophenarsine into dogs, the drug does not circulate as such for more than three to six hours. On the other hand, a closely related phenylarsenoxide containing a p-CONH<sub>2</sub> instead of a p-OH group has greater stability and may exist as such for 24 hours. In the case of oxophenarsine, the observed facts are consistent with the hypothesis that the arsenic-carbon bond is cleaved and that the resulting fractions are o-aminophenol and As<sub>2</sub>O<sub>3</sub> or As<sub>2</sub>O<sub>5</sub>. Chemically, this cleavage has been accomplished, and o-aminophenol and arsenious oxide isolated (13). The formation of inorganic arsenic would explain the results of Wright and Peters (4) and those communicated to the present author by Banks (14). In those experiments, using rats, it was found that six hours after injection of oxophenar-



sine the trypanocidal titer of the plasma decreased, although arsenic levels were maintained or increased. Inorganic arsenic has far less antiparasitic effect than do the phenylarsenoxides. The link between the aromatic ring and arsenic is essential for therapeutic utility in infectious diseases. Organic or inorganic arsenic may be bound to thiol proteins *in vivo* (16, 17); binding of the intact oxophenarsine to a dithiol, however, does not eliminate its trypanocidal action (18). The more significant detoxification and loss of activity may therefore be that associated with rupture of the arsenic-carbon bond.

It should be mentioned, however, that Crawford and Levvy (15) found no evidence that unsubstituted phenylarsenoxide was degraded to  $As_2O_3$  or  $As_2O_5$  in the rabbit. In a careful study of phenylarsenoxide, they found that 50 per cent was oxidized to the corresponding arsonic acid, and that the remainder could not be accounted for.

Whatever the alteration is, the present work indicates that oxophenarsine is not stable in the dog. It is probable that it is converted into a compound or compounds that are less active therapeutically than the parent.

If m-amino-p-carbamylphenylarsenoxide is less rapidly destroyed in the body than oxophenarsine, it may be of interest to reconsider the use of this compound in syphilis as an adjunct to penicillin. Its toxicity and therapeutic activity in *T. pallidum* in rabbits is approximately the same as that of oxophenarsine (19), and the pharmacological difference indicated here could conceivably be of therapeutic advantage in man. Eagle has shown that the antispirochetal efficacy of metallic compounds increases as exposure to the organism is prolonged (20). If concentrations in urine and plasma are indicative, the substitution of a p-CONH<sub>2</sub> for a p-OH group in oxophenarsine would considerably extend the period of exposure following single injection. If this resulted in a shortening of the dosage schedule (that for oxophenarsine is 26 weeks) and reduction of the total drug administered, it would be of considerable practical value.

Side chains in the series of phenylarsenoxides have, in the past, been shown to lend specificity to the treatment of experimental and human infections. This is the basis for the use of the p-(CH<sub>2</sub>)<sub>3</sub>COOH derivative in trypanosomiasis (21), and the p-CONH<sub>2</sub> compound in filariasis (22). The present study suggests that side chains are also involved in the stability of these compounds *in vivo*.

#### SUMMARY

1. A colorimetric method for the determination of o-aminophenols has been described. This has been applied to plasma and urine to determine the o-aminophenol component after the administration of oxophenarsine to dogs. Parallel determinations of the arsenic content were performed.

2. Oxophenarsine was found to be relatively unstable in the dog, existing as such for only three to six hours.

3. Similar studies were carried out for m-amino-p-carbamylphenylarsenoxide, using determinations of arylamino group and arsenic. The results suggest that this compound is relatively stable, and may circulate as such for 12-24 hours.

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## REFERENCES

1. EAGLE, H.: *Am. J. Syph. Gonorr. & Ven. Dis.*, **23**: 310, 1939.
2. MAGNUSON, H. J., AND RAULSTON, B. O.: *Ann. Int. Med.*, **14**: 2199, 1940.
3. HAWKING, F.: *THIS JOURNAL*, **59**: 157, 1937.
4. WRIGHT, H. N., AND PETERS, L.: *Proc. Soc. Exper. Biol. & Med.*, **52**: 3, 1943.
5. BEESON, B. B., AND ALBRECHT, P. G.: *Arch. Dermat. & Syph.*, **5**: 51, 1922.
6. AUTENRIETH, W., AND TAEGER, H.: *München med. Wehnschr.*, **69**: 1479, 1922.
7. KOLLS, A. C., AND YOUNG, A. C.: *Bull. Johns Hopkins Hosp.*, **34**: 149, 1932.
8. BRATTON, A. C., JR., AND MARSHALL, E. K., JR.: *J. Biol. Chem.*, **128**: 537, 1939.
9. MARSHALL, E. K., JR.: Personal communication.
10. MAREN, T. H.: *Ind. Eng. Chem., Analytical Edition*, **18**: 521, 1946.
11. MAGNUSON, H. J., AND WATSON, E.: *Ind. Eng. Chem., Analytical Edition*, **16**: 339, 1944.
12. GRUHZIT, O. M., SULTZBERGER, J. A., AND SHAFER, L. W.: *Arch. Dermat. & Syphil.*, **49**: 321, 1944.
13. CHRISTIANSEN, W. G.: *J. Am. Chem. Soc.*, **43**: 370, 1921.
14. Personal communication, reporting work from the Parke, Davis Laboratories.
15. CRAWFORD, T. B. B., AND LEVY, G. A.: *Biochem. J.*, **41**: 333, 1947.
16. VOEGTLIN, C., DYER, H. A., AND LEONARD, C. S.: *U. S. Public Health Reports*, **38**: 1882, 1923.
17. PETERS, R. A.: *Nature*, **156**: 616, 1945.
18. SAWYERS, J. L., BURROWS, B., AND MAREN, T. H.: *Proc. Soc. Exper. Biol. & Med.* in press.
19. EAGLE, H., HOGAN, R. D., DOAK, G. O., AND STEINMAN, H. G.: *THIS JOURNAL*, **61**: 142, 1944.
20. EAGLE, H.: *Am. J. Syph. Gonorr. & Ven. Dis.*, **23**: 310, 1939.
21. EAGLE, H.: *U. S. Public Health Reports*, **61**: 1019, 1946.
22. OTTO, G. F., AND MAREN, T. H.: *Am. J. Hygiene*, in press.



# THE INFLUENCE OF THE DOSAGE REGIMEN ON THE THERAPEUTIC ACTIVITY OF PENICILLIN G<sup>1</sup>

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Until recently it has been assumed generally that the therapeutic effectiveness of the penicillins depends upon their administration in such a way that contacts between drug and invading organisms are constantly maintained. This assumption coupled with knowledge of the rapid elimination of the penicillins has dominated ideas on the practical use of these substances. Thus all major schemes of treatment, which have been favored since inception of penicillin therapy,<sup>2</sup> seem to have been designed with the objective of continually maintaining the plasma or serum concentrations of these antibiotics at or above levels which are known to inhibit growth of the invading organisms.

As Marshall (1) has pointed out, examination of the background of the above concept shows that it has been conditioned more by precepts accepted in the field of sulfonamide therapy than by established fact. Certainly, there have been few clinical data which would support the belief that the effectiveness of penicillin therapy is dependent upon the continued maintenance of inhibitory concentrations of these antibiotics in blood plasma. Evidence in apparent opposition to this belief was available as early as 1944 and 1945. At that time, studies by Tillett and co-workers (2, 3) with rather crude preparations of the penicillins indicated that pneumococcal lobar pneumonia could be treated satisfactorily with widely spaced doses of these drugs—doses which could not have maintained inhibitory concentrations of the penicillins in the body fluids throughout the treatment intervals. More recently, reports (4-8) dealing with a variety of human infections have proved the efficacy of 12- to 24-hourly administration of aqueous solutions of highly purified sodium penicillin G.

Although the above findings refute the belief that the constant presence of critical concentrations of the penicillins is essential to satisfactory therapeutic responses, the data currently available do not permit definite conclusions on the relative merits of frequent and widely spaced treatments. Since, for obvious reasons, it may be extremely difficult to reach such conclusions through studies in severely ill patients, where data would be most significant, studies on the effectiveness of various dosage regimes against infections in experimental animals are especially valuable. Several such studies (9-13) have already been made but have yielded apparently conflicting results. Although there are some obvious differences in the experimental techniques used in these studies and

<sup>1</sup> This work was supported in part by the Parke G. Smith Urology Fund.

<sup>2</sup> These include in chronological order continuous intravenous infusion, intramuscular injections at intervals of two to four hours, administration of repository preparations at less frequent intervals, and blocking of renal clearance with agents such as caronamide.

some of the supporting data are meager (12, 13), there appears to be no simple explanation for their divergent findings. The question of the influence of the frequency of administration on the therapeutic effectiveness of the penicillins certainly requires more comprehensive investigation.

Studies on the relationship between the therapeutic effectiveness of penicillin G and the dosage regimen were initiated in this laboratory in November 1947. The type of infection selected for this work was the rapidly developing, widely disseminated disease which follows intraperitoneal inoculation with pneumococci. The rat was selected as the experimental animal with the idea of later studying in this same host the dosage regimen-therapeutic effect relations which prevail in the more slowly developing, comparatively localized lobar pneumonia. The results of the studies on the rapidly developing, generalized infection are reported here.

#### EXPERIMENTAL

##### *A. Effectiveness of different dosage regimens:*

**METHODS.** Male rats, Sprague-Dawley strain, were used in this study. The animals were seven to nine weeks old at the time of inoculation and ranged in weight from 125 to 180 grams.

The McGovern strain of type I pneumococcus was used as the infecting organism. This strain has well stabilized characteristics, having been passaged through normal mice either daily or on alternate days for more than ten years. The virulence of this strain for the rat was determined repeatedly during the course of the present study. On each occasion, intraperitoneal inoculation with one to five organisms produced uniformly fatal infections, death occurring within 20 to 48 hours. The inocula employed in the therapeutic experiments were obtained through suitable dilution in infusion broth of 8-hour subcultures prepared from routine mouse passage cultures. All inoculations were made via the intraperitoneal route, using 1.0 cc. of a  $10^{-8}$  dilution of the above subculture. These inocula, which contained from 1,000 to 15,000 pneumococci, proved fatal to all 266 untreated control rats, the average survival time being 29 hours, S.D.  $\pm 4.04$ .

The crystalline sodium salt of penicillin G was used in all but one series of therapeutic experiments. This material, of a single lot, was obtained from the Commercial Solvents Company, and had a stated potency of 1620 units/mgm. Insofar as could be determined by systematic comparisons in the infected rat, the therapeutic activity of this preparation was identical with that of a reference sample of sodium penicillin G provided by the Antibiotics Study Section of the U. S. Public Health Service. This reference lot, No. 2846 C, contained more than 99 per cent sodium penicillin G according to Craig counter-current distribution analysis and had a potency of 1667 units/mgm.\* In one series of experiments, procaine penicillin G was employed as the therapeutic agent. The preparation used, Crysticillin, E. R. Squibb and Sons, was also of one lot. The dose of this material was calculated in terms of sodium penicillin G equivalent, assuming that the penicillin G content of the procaine penicillin was correct as stated.

Throughout this investigation, therapy was initiated four hours after intraperitoneal inoculation. A study with some 80 untreated control rats demonstrated that blood cultures were uniformly positive at this time and that the bacteremias ranged from 2,000 to 80,000 organisms/cc. Thus a widely disseminated infection was present at the time therapy was started.

\* Information on the purity and activity of the reference sample of sodium penicillin G was provided through the courtesy of Dr. Gordon Seeger, National Institutes of Health, Bethesda, Maryland.

Nine different dosage schedules were employed in the experiments with sodium penicillin G, four of short duration, five of longer duration. Treatments of short duration included a single dose schedule and three schedules of 2 doses separated by 2, 4, or 8 hours. The regimens of longer duration, such as might be encountered in clinical practice, covered the equivalent of four days and included 48, 24, 12, 8, and 4 doses at 2-, 4-, 8-, 12-, and 24-hour intervals, respectively. In the experiments with procaine penicillin G, four doses were administered at 24-hour intervals. In all cases, the appropriate amount of penicillin, dissolved or suspended in 0.25 cc. of sterile 0.85 per cent saline, was injected into the thigh muscles of the rear leg.

Rats which did not succumb to the infection were kept under observation for 21 days. Necropsies and cultures of heart blood were made on representative animals which died after end of treatment. In selected cases, cultures of tail blood were prepared in the interim between the termination of therapy and the end of the observation period. Whenever accumulations of pus or fluid were noted in the abdominal and thoracic cavities, or whenever there was evidence of meningitis, cultures of the exudate or spinal fluid were prepared and studied.

TABLE 1  
*Reproducibility of results in different experiments with sodium penicillin G*

DOSAGE REGIME	DATE OF EXPERIMENT	NO. SURVIVORS/NO. RATS INFECTED		
		Total dose sodium penicillin G—mgm./kgm. body weight		
		4	8	16
12 doses at 8-hour intervals	4-20-48	1/24	9/24	
	5-4 -48	3/24	5/24	19/24
	8-3 -48			19/24
24 doses at 4-hour intervals	6-3 -48		5/24	24/24
	6-15-48		9/24	24/24
	9-7 -48		9/24	24/24

The procedures used in assessing the effectiveness of various dosage regimes were briefly as follows. Preliminary experiments were carried out with each dosage schedule, using six rats for each of some six to ten doses of penicillin. Depending upon the results of this experiment, a series of four or five doses was selected which for a given treatment regime would be expected to afford from 20 to 100 per cent protection. Then a major experiment was performed, involving from 90 to 104 rats with at least 8 untreated control animals and 4 or 5 groups of 16 to 24 rats treated with the range of penicillin doses indicated above. With almost every dosage schedule, supplementary experiments were carried out with doses of penicillin which appeared to offer between 30 and 80 per cent protection. The data presented in the following section of this report include the results of the original major experiment and all supplementary tests.

Since it was not practical to work with all different dosage regimes at one time, attention should be called to the reproducibility of results when the same dosage regime was used at different times. Representative data for two dosage regimes, presented in table 1, show that remarkably similar results were obtained when the same doses of drug were employed in different experiments.

RESULTS. Table 2 summarizes the data obtained with the various dosage regimes of penicillin G. The data presented in the upper section of this table

TABLE 2

*The influence of the dosage regimen on the therapeutic activity of penicillin G*

NO. OF DOSES AND DOSAGE INTERVAL*	DOSE OF SODIUM PENI- CILLIN G, MCG./KG. BODY WEIGHT		NO. SURVI- VORS/NO. RATS IN- FECTED	SURVI- VORS	AVERAGE SURVIVAL OF RATS THAT DIED	ED <sub>50</sub> AND STAND- ARD ERROR†	APPROXI- MATE ED <sub>50</sub> ‡
	Total	Individual treatment					
Therapy with sodium penicillin G—short duration							
Single dose	8	8	4/46	9	51	48 ± 11.6	>128
	16	16	7/46	15	51		
	32	32	21/46	46	52		
	64	64	25/46	54	55		
	128	128	16/24	67	77		
2 doses at 2-hour intervals	4	2	5/24	21	56	16 ± 2.8	64
	8	4	13/48	27	63		
	16	8	13/24	54	73		
	32	16	17/24	71	89		
	64	32	23/24	96	72		
2 doses at 4-hour intervals	2	1	6/24	25	82	4 ± 0.35	8
	4	2	28/48	58	76		
	8	4	22/24	92	60		
	16	8	21/24	88	72		
	32	16	24/24	100	—		
2 doses at 8-hour intervals	16	8	9/48	19	63	56 ± 8.0	256
	32	16	7/48	15	66		
	64	32	15/24	63	67		
	128	64	18/24	75	74		
	256	128	23/24	96	72		
Therapy with sodium penicillin G—4 days duration							
48 doses at 2-hour intervals	4	0.083	3/24	13	127	10 ± 0.41	16
	8	0.167	11/48	23	235		
	16	0.333	45/48	94	388		
	32	0.667	24/24	100	—		
24 doses at 4-hour intervals	2	0.083	1/24	4	34	10 ± 0.34	16
	4	0.167	2/24	8	101		
	8	0.333	23/72	32	199		
	16	0.667	72/72	100	—		
12 doses at 8-hour intervals	2	0.167	1/24	4	82	10 ± 0.77	32
	4	0.333	4/48	8	138		
	8	0.667	14/48	29	192		
	16	1.33	38/48	79	158		
	32	2.67	22/24	92	228		
8 doses at 12-hour intervals	8	1	4/48	8	191	80 ± 9.2	>128
	16	2	3/24	13	227		
	32	4	4/24	17	201		
	64	8	18/48	38	205		
	128	16	37/48	77	196		

TABLE 2—Continued

NO. OF DOSES AND DOSAGE INTERVAL*	DOSE OF SODIUM PEN- ICILLIN G, MCG./KGM. BODY WEIGHT		NO. SURVI- VORS/NO. RATS IN- FECTED	SURVI- VORS	AVERAGE SURVIVAL OF RATS THAT DIED	ED <sub>50</sub> AND STAND- ARD ERROR†	APPROXI- MATE ED <sub>90</sub> ‡
	Total	Individual treatment					
				<i>per cent</i>	<i>hours</i>	<i>mgm./kgm.</i>	<i>mgm./kgm.</i>
4 doses at 24-hour intervals	16	4	5/48	11	164	175 ± 26	>256
	32	8	9/70	13	174		
	64	16	1/60	2	198		
	128	32	26/72	36	216		
	256	64	46/72	64	204		
Therapy with procaine penicillin G—4 days duration							
4 doses at 24-hour intervals	16	4	5/32	16	102	44 ± 5.6	128
	32	8	18/56	32	163		
	64	16	40/56	71	207		
	128	32	24/24	100	—		
Untreated controls							
—	—	—	0/266	0	29	—	—

\* In all dosage regimes, the first dose of penicillin G was administered 4 hours after infection.

† Calculations according to method of Litchfield and Fertig (14) as modified by Miller and Tainter (15).

‡ Approximated from raw data as lowest dose curing 90 per cent or more of the animals.

show clearly that, with treatment limited to a brief period, two doses of sodium penicillin G administered four hours apart gave considerably better results than a single dose of drug or two doses separated by an interval of two or eight hours. The ED<sub>50</sub> values for the regimes indicated above were 4, 48, 16, and 56 mgm./kgm., respectively, while the approximate ED<sub>90</sub> values were 8, >128, 64, and 256 mgm./kgm. From these data it is also clear that two doses administered two hours apart were more effective than two doses separated by an 8-hour interval. The latter regime was in no significant way superior to a single dose of drug.

With treatment of longer duration, covering a 4-day period, 2-, 4-, or 8-hourly administration of sodium penicillin G proved strikingly more effective than administration at 12- or 24-hour intervals. As judged by the total doses required for both 50 and 90 per cent protection, best results were obtained when penicillin G was administered at intervals of two or four hours. These two regimes were equal in effectiveness in all respects, the ED<sub>50</sub>s and ED<sub>90</sub>s being 10 and 16 mgm./kgm., respectively. Repeated treatments at 8-hour intervals were only slightly less effective than treatments at two and four hours. The ED<sub>50</sub> on the 8-hour regime was the same as ED<sub>50</sub>s for the 2- and 4-hour treatments; however, the

ED<sub>90</sub> on the 8-hour schedule was somewhat greater. When the intervals between treatments were extended to 12 or 24 hours, the effectiveness of sodium penicillin G therapy decreased markedly. On these regimes the ED<sub>50</sub>s were 80 and 175 mgm./kgm., respectively, while the approximate ED<sub>90</sub>s were considerably in excess of 128 and 256 mgm./kgm. When the treatments, administered over a 96-hour period, were separated by intervals of 24 hours, curative results were no better than when  $\frac{1}{4}$  of this total dose was given in a single treatment. However, the multiple doses did prolong life to a significantly greater degree.

It should also be mentioned that the slopes of the dosage response curves were considerably steeper when treatments were administered at 2- or 4-hour intervals than when given at less frequent intervals. Thus there was a progressive spread between the doses required for 50 and 90 per cent protection as the treatment interval increased from 4 to 24 hours.

Data on the effectiveness of treatment with procaine penicillin G, presented in the lower section of table 2, show clearly that this slowly absorbed preparation, administered at intervals of 24 hours, was much less effective than the more rapidly absorbed sodium penicillin administered at intervals of 2, 4, or 8 hours. However, treatment with procaine penicillin at 24-hourly periods was considerably more effective than treatment with the sodium salt at intervals of 12 or 24 hours.

Special attention should be drawn to the clinical courses of the disease in infections which were not cured by the different treatment regimes. Where therapy was of short duration, animals were outwardly in excellent health for a period of 24 to 48 hours after the last treatment. Their fur then became ruffled, generalized weakness developed, and death with an overwhelming bacteremia followed within another 24 hours. From the onset of symptoms until death, the clinical course in these rats was much like that of untreated controls.

Some of the rats scheduled for 4-day treatment with penicillin died during the period of therapy or shortly thereafter. In these cases, illness was usually apparent within 24 hours after inoculation; thereafter, the animals exhibited a steadily downhill course with a gradually increasing bacteremia. Other rats, which received subcurative doses of penicillin for four days, died many days after the end of treatment, exhibiting an entirely different clinical course from that just described. Such animals were outwardly in excellent health and had negative blood cultures at the end of treatment. Two to eight days later a remarkable series of reactions occurred. Some animals developed meningitis and remained in coma for nearly a week before succumbing; others developed large walled-off intra-abdominal accumulations of purulent fluid containing innumerable pneumococci. At times more than one such abscess was present in the same animal. Other rats exhibited a very gradual downhill course without any of the symptoms mentioned above; necropsies invariably revealed extensive accumulations of purulent fluid within the pericardium or pleural cavity. This fluid, like that in the abdomen, contained innumerable pneumococci. Cultures, prepared from the tail blood of representative rats exhibiting the above syndromes, indicated that pneumococci were present in the blood from the onset

of symptoms. Except in the terminal stages, however, the bacteremias were comparatively low. The sequence of events in such animals suggests that a humoral immunity to the organism, acquired during penicillin therapy, sufficed to prevent development of an overwhelming generalized infection but could not prevent growth of pneumococci in isolated regions.

The incidence of the above complications was not related to a particular dosage regime and was essentially the same among all rats which survived 4-day treatment schedules but succumbed late in the post-treatment observation period.

*B. Concentrations of "penicillin" in plasma during the various dosage regimes:*

**METHODS.** In order to evaluate more critically the data presented in the preceding section, studies were made of the concentrations of "penicillin" (bacteriostatic substances)

TABLE 3  
Concentrations of "penicillin" in plasma following administration of  $ED_{50}$ s obtained for various dosage regimes

HOURS AFTER TREATMENT	MICROGM. "PENICILLIN"/CC. PLASMA					
	Equivalent dose of sodium penicillin G—mgm./kgm. body weight					
	0.25*	0.5*	1.0*	12*	64*	12†
$\frac{1}{2}$	0.12	0.12	0.24	7.68	30.72	3.84
$\frac{1}{4}$	0.06	0.06	0.12	3.84	15.36	1.92
1	0.03	0.03	0.06	0.48	7.68	1.92
2	<0.015	<0.015	<0.015	0.12	0.96	0.96
4		<0.015	<0.015	<0.015	0.03	0.48
6			<0.015	<0.015	<0.015	0.12
8			<0.015	<0.015	<0.015	<0.015
12				<0.015	<0.015	<0.015
24					<0.015	<0.015

\* Amounts of sodium penicillin G corresponding respectively to  $ED_{50}$ s for 2-, 4-, 8-, 12-, and 24-hourly regimes.

† Amount of procaine penicillin G (equivalent to sodium salt) corresponding to  $ED_{50}$  for 24-hourly regime.

which were present in blood plasma at various intervals following administration, via the different dosage regimes, of equally effective amounts of sodium or procaine penicillin G. The doses selected for this experiment were the individual treatment fractions of total doses which, according to the data in table 2, provided 50 per cent protection ( $ED_{50}$ ). Each of the doses (0.25, 0.5, 1, 12, and 64 mgm./kgm. for the 2-, 4-, 8-, 12-, and 24-hourly schedules with sodium penicillin G, and 12 mgm./kgm. for the 24-hourly regime with procaine penicillin) was administered intramuscularly to a group of ten rats, males, 9 to 10 weeks old, weighing 180 to 220 gm. Heart blood samples, 0.5 cc., were obtained from each animal within a group at intervals ranging from  $\frac{1}{2}$  to 24 hours after administration of penicillin. These samples, treated with heparin, were pooled and centrifuged immediately after collection. The resulting plasma was assayed for "penicillin" content by a slight modification of the method of Rammelkamp (16), using the C-203 stain of streptococcus as the test organism. Assays were run in duplicate; in most instances, experiments with given doses were performed twice. It should be noted here that the least quantity of penicillin G which could be measured was 0.015 microgm. (0.025 units)/cc. of plasma, also that the

activity of penicillin G was not inhibited by the concentrations of rat plasma employed in this assay procedure.

**Results.** The data obtained in the above experiments, summarized in table 3, confirm the well-known facts that the height of the "penicillin" level in plasma is directly related to the dose of drug and that peak levels are attained within  $\frac{1}{4}$  to  $\frac{1}{2}$  hour after intramuscular administration of sodium penicillin.

It is noteworthy that none of the doses, which provided 50 per cent protection against pneumococcal infection, maintained measurable amounts of "penicillin" in plasma throughout the intervals between treatments. Measurable levels were present only one hour after administration of sodium penicillin G in doses of 0.25, 0.5, and 1.0 mgm./kgm., the amounts which proved effective on the 2-, 4-, and 8-hourly dosage schedules. The dose which was effective on the 12-hourly schedule provided measurable levels for only two hours, while the 24-hourly dose gave measurable levels for four hours. Procaine penicillin produced better sustained levels than the same dose of sodium penicillin but, even with this "long acting" preparation, measurable concentrations were present for only six hours.

It is important to note that in any one of the so-called effective dosage regimes there was a considerable portion of the dosage interval in which the concentration of penicillin in plasma was below the level which would inhibit the growth of the McGovern strain of pneumococcus.<sup>4</sup> These "penicillin-free" periods were respectively 1, 3, 7, 10, and 20 hours on the 2-, 4-, 8-, 12-, and 24-hourly schedules with sodium penicillin, and 18 hours on the 24-hourly schedule with procaine penicillin.

**Discussion.** The data in this report show conclusively that cure of a high proportion of fulminating pneumococcal infections can be accomplished by administration of sodium penicillin G at either frequently spaced or widely spaced intervals. It is equally clear that, from a quantitative point of view, the effectiveness of this antibiotic is related to the frequency with which the drug is administered. The optimum treatment interval is determined to some extent, however, by the duration of therapy. Thus when sodium penicillin G was administered for a very brief period (eight hours or less) two doses, four hours apart, provided optimum results and gave significantly greater protection than two doses at intervals of two or eight hours. On the other hand, with a treatment period of approximately 96 hours, comparable to what is frequently employed in clinical practice, 2-, 4-, and 8-hour dosage schedules were essentially equal in effectiveness. Each of these regimes was eight times as effective as a 12-hour schedule and seventeen times as effective as a 24-hour schedule.

This conclusion, that the effectiveness of sodium penicillin G therapy is related to the frequency with which this drug is administered, is in accord with conclusions derived from the studies of Jawetz (9) and Eagle (12) who worked

<sup>4</sup> Previous studies have shown that 0.03 microgm./cc. is the lowest concentration of sodium penicillin G which will regularly inhibit growth of the McGovern strain *in vitro*. This is twice the minimal concentration which can be measured by the assay procedure used in the present experiments.



respectively with streptococcal and pneumococcal infections in mice. It is opposed, however, to the conclusions drawn from the studies of Zubrod (10) and White (11) on streptococcal infections and those of Gibson (13) on pneumococcal infections. The latter investigators, also employing the mouse as the test animal, found that the effectiveness of various water-soluble penicillins was related to the total dose of drug rather than to the frequency with which individual doses were administered. It is apparent that these opposing conclusions cannot be ascribed simply to the use of different species of test organisms or different experimental animals. Some of the above studies may be criticized for incomplete documentation of conclusions (12), and use of crude penicillin preparations (9), inadequate numbers of experimental animals (13), or infecting organisms of uncertain virulence (9). However, divergent conclusions were also attained in studies which appear to be free of such criticisms, namely in the work of Zubrod, White, and this laboratory. It seems obvious, therefore, that there must be basic differences in the characteristics of infections produced by different organisms, which determine the relative activities of frequent and widely spaced dosage regimes.

It appears probable that these differences rest on inherent differences in the susceptibility of the invading organisms to the defense reactions of the host. It is well-known that various species and strains of bacteria differ widely in this respect. Such differences are probably far more important to successful penicillin therapy than is commonly recognized. Undoubtedly, penicillin does not kill every one of the infecting organisms even under optimum conditions. Although a very high proportion of the organisms may be destroyed by this bactericidal agent, some few either receive sublethal injury or are essentially unaffected because of their existence in a resting state. If these persisting bacteria are not removed by the phagocytes or killed by repeated exposure to penicillin, they ultimately overwhelm the host. The success of the single dose regimes of Zubrod and White and the widely spaced doses of Gibson probably depends on the fact that those few organisms of the C-203 strain of streptococcus and the Gibson strain of pneumococcus, which were not killed directly by the penicillin, were readily disposed of by the phagocytes of the mouse. On the other hand, the insusceptibility to phagocytosis of the Jawetz strain of streptococcus and the McGovern strain of pneumococcus placed responsibility for eradicating these organisms on the penicillin; in this circumstance repeated administration of the drug was required to eradicate all of the invading bacteria.

There is a certain amount of evidence to support the suggestion that the effectiveness of various dosage regimes of penicillin depends, in part, upon the susceptibility of specific organisms to body defense mechanisms. Work in this laboratory on problems of drug resistance (17) has shown that much larger total doses of penicillin and more protracted treatment are required to cure mouse infections with the McGovern strain of pneumococcus than are required for infections with the C-203 strain of streptococcus. MacLeod and Stone (18) likewise found that there were marked differences in the amounts of penicillin and duration of treatment required to cure infections with various strains of type I,

II, and III pneumococcus. Since the organisms used in both of the above studies had essentially equal virulence and *in vitro* susceptibility to the penicillins, the variations in *in vivo* responses were probably due to differences in the ability of the mouse phagocytes to dispose of those few bacteria which were not killed directly by exposure to the drug.

The present study has also raised some interesting points on the relations between the effectiveness of penicillin therapy and the levels of this drug in plasma. The data presented here show that sustained plasma concentrations of penicillin G are not at all necessary for a maximum therapeutic response. Periods in which the plasma was "penicillin-free" varied from one to twenty hours on various dosage regimes of equal effectiveness; these periods covered from  $\frac{1}{2}$  to  $\frac{5}{8}$  of the total dosage intervals. It is noteworthy that, by increasing the dose, the "penicillin-free" period can be greatly prolonged without loss of therapeutic effect, also that those regimes which had the longest "penicillin-free" periods are associated with the highest plasma levels of drug. This latter finding is of especial interest. It indicates that plasma penicillin concentrations above levels required to kill organisms at so-called maximal rates have a definite beneficial effect and are not so much "waste drug" as has been maintained by Eagle (12). These high concentrations may either lengthen the lag period in the recovery of organisms which have been exposed to toxic but sublethal concentrations of penicillin (19) or effect a greater localization of the drug in the invading bacteria.

It is always necessary to exercise great conservatism in transferring the results of an experimental study to clinical use. Differences in disposition of the therapeutic agent, in infecting organisms, in specific characteristics of the infections, and in the inherent susceptibility of the host to disease processes, all serve as modifying factors. It seems reasonable to conclude, however, from the results of the present and previous studies that optimum therapeutic responses in fulminating human infections with such organisms as the pneumococcus and streptococcus can be achieved by administration of sodium penicillin G no more frequently than every eight hours, and probably somewhat less frequently because of the slower elimination of penicillin in the human patient. Much less frequent dosage may be required for optimum effects when one is dealing with more or less circumscribed infections. It also is reasonable to conclude that the continued presence of inhibitory concentrations of penicillin G in plasma is not essential to a maximum therapeutic response. These conclusions would eliminate the alleged basis for the general clinical use of repository preparations of the penicillins as well as for the 3- to 4-hourly and intravenous treatment schedules.

#### SUMMARY

A quantitative study has been made of the effectiveness of different dosage regimes of penicillin G against a fulminating pneumococcal infection in the white rat. The results of this study have shown that with treatment of brief duration (eight hours or less) two doses of sodium penicillin G administered two or four hours apart were significantly more effective than a single dose or two doses

separated by an 8-hour interval. Likewise, when treatment was of moderate duration (96 hours or the equivalent), divided doses of sodium penicillin G administered at 2-, 4-, or 8-hour intervals were far more effective than divided doses at 12- or 24-hour intervals. It was also found that procaine penicillin G administered at 24-hour intervals was significantly more effective than sodium penicillin G given at these times, but not as effective as the sodium salt administered at 2-, 4-, or 8-hour intervals.

Measurements were also made of the concentrations of "penicillin" present in plasma after administration of equally effective doses of sodium or procaine penicillin G on the various dosage regimes. These measurements show that these equally effective doses maintained inhibitory concentrations of "penicillin" in plasma for only a small fraction of the dosage interval.

Although the above findings show that the effectiveness of penicillin therapy is related to the frequency of treatment, they offer no support for the general assumption that the effectiveness of therapy is dependent upon continued maintenance of inhibitory concentrations of the drug in body fluids. Rather they indicate that optimum therapeutic results, even in fulminating infections, may be achieved by administration of water-soluble penicillin salts at fairly widely spaced intervals.

#### REFERENCES

- (1) MARSHALL, E. K., JR.: Bull. Johns Hopkins Hosp., 82: 403, 1948.
- (2) TILLET, W. S., CAMBIER, M. J., AND McCORMACK, J. E.: Bull. New York Acad. Med., 20: 142, 1944.
- (3) TILLET, W. S., CAMBIER, M. J., AND McCORMACK, J. E.: J. Clin. Investigation, 24: 589, 1945.
- (4) JAWETZ, E.: Arch. Int. Med., 81: 203, 1948.
- (5) TUMULTY, P. A., AND ZUBROD, G.: New England J. Med., 239: 1033, 1948.
- (6) ALTEMEIER, W. A.: Arch. Surg., 57: 396, 1948.
- (7) WEISS, W., AND STEINBERG, I.: Am. J. M. Sc., 217: 86, 1949.
- (8) HAMBURGER, M., BERMAN, J. R., THOMPSON, R. T., AND BLANKENHORN, M. A.: J. Lab. and Clin. Med., 34: 59, 1949.
- (9) JAWETZ, E.: Arch. Int. Med., 77: 1, 1946.
- (10) ZUBROD, C. G.: Bull. Johns Hopkins Hosp., 81: 400, 1947.
- (11) WHITE, H. J., BAKER, M. J., AND JACKSON, E. R.: Proc. Soc. Exper. Biol. and Med., 67: 199, 1948.
- (12) EAGLE, H.: Ann. Int. Med., 28: 260, 1948.
- (13) GIBSON, C. D., JR.: Proc. Soc. Exper. Biol. and Med., 67: 278, 1948.
- (14) LITCHFIELD, J. T., JR., AND FERTIG, J. W.: Bull. Johns Hopkins Hosp., 69: 276, 1941.
- (15) MILLER, L. C., AND TANTER, M. L.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
- (16) RAMMELKAMP, C. H.: Proc. Soc. Exper. Biol. and Med., 51: 95, 1942.
- (17) SCHMIDT, L. H., AND SESLER, C. L.: unpublished observations.
- (18) MACLEOD, C. M., AND STONE, E. R.: Bull. New York Acad. Med., 21: 375, 1945.
- (19) PARKER, R. F., AND MARSH, H. C.: J. Bact., 51: 181, 1946.

# THE PREPARATION AND PHARMACOLOGICAL PROPERTIES OF THE ACID SUCCINATE OF 3-(o-TOLOXY)-1,2-PROPANEDIOL (MYANESIN)<sup>1</sup>

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The compound, 3-(o-toloxyl)-1,2-propanediol, variously called Myanesin, Tolserol, Toloxyn, etc., has been shown to produce muscular relaxation during light anesthesia (1-6) and to influence favorably various dystonic and hyperkinetic states (7-10). The practical value of the drug has been limited, however, because of its low solubility in water and the short duration of its action. The examination of a large number of various 3-alkyloxy and 3-alkyl-aryloxy-1,2-propanediols and 3-alkylarylthio-1,2-propanediols did not bring to light any compound of greater solubility or longer duration of action (11). The duration of action of all the active compounds was similar, a fact which suggested that the body may rapidly inactivate all of these compounds by a similar mechanism.

Metabolic studies have shown that very little free Myanesin is excreted unchanged in the urine (12-15). Further studies by two groups working independently have disclosed that beta-(o-toloxyl)-lactic acid, in which the terminal group of Myanesin has been oxidized from  $-\text{CH}_2\text{OH}$  to  $\text{COOH}$ , is an important metabolic product of the drug (16, 17). This metabolite is a true detoxification product devoid of paralyzing properties and of low toxicity (16). Thus it appears that the presence of the terminal alcohol group of Myanesin is essential for activity.

The demonstration that a major part of the Myanesin molecule is rendered ineffective by a rapid oxidative process suggested the possibility of prolonging Myanesin action by protecting the hydroxyl group involved. Such a compound might be more soluble in water than is Myanesin and it was expected that such a compound would be relatively non-toxic and would liberate the active parent compound *in vivo*. The present report describes the chemical and pharmacological properties of the first such compound prepared and studied, the acid succinate of 3-(o-toloxyl)-1,2-propanediol. The assumptions were well-founded; Myanesin acid succinate formed a very soluble sodium salt which possessed Myanesin-like activity. The compound was less toxic and produced effects of longer duration than Myanesin.

## EXPERIMENTAL

1. PREPARATION OF MYANESIN ACID SUCCINATE (MAS). The acid succinate of 3-(o-toloxyl)-1,2-propanediol was prepared by the direct succinylation of Myanesin. One

<sup>1</sup> Aided by a grant from the National Foundation for Infantile Paralysis.

half mol (92 gm.) of the diol and 50 gm. of succinic anhydride were dissolved in 225 cc. pyridine and the mixture heated overnight on the steam bath. The reactants were cooled, acidified with 3 *N* HCl and extracted with ether. Extraction of the ether solution with 8 per cent sodium bicarbonate transferred the succinate to the aqueous solution which was then washed with several portions of fresh ether to insure the removal of any unreacted Myanesin. Acidification of the aqueous solution of the sodium salt precipitated the succinate as a viscous oil which was again taken up in ether. Evaporation of the solvent under high vacuum left the succinate as a viscous, straw-colored liquid which has resisted all attempts at crystallization. Yield 86 per cent of theory. Calculated for  $C_{14}H_{18}O_6$ : C, 59.56; H, 6.43; Neut. eq., 282.3. Found: C, 58.76; H, 6.51; Neut. eq., 279.0; 279.5.

The succinate is soluble in ether, alcohol, acetone, benzene, and chloroform but is only slightly soluble in water or petroleum ether. For physiological testing, the succinate was dissolved in sufficient sodium hydroxide to give a 10 per cent aqueous solution with a pH

TABLE 1

*Incidence of paralysis and death in mice after intraperitoneal administration of various doses of aqueous solutions of the acid succinate of 3-(o-toloxyl)-1,2-propanediol and of Myanesin*

DOSE	ACID SUCCINATE OF 3-(O-TOLOXY)- 1,2-PROPANEDIOL		MYANESIN	
	No. paralysed No. used	No. died* No. used	No. paralysed No. used	No. died* No. used
mM/kgm.				
0.7	—	—	0/10	3/10
1.0	0/10	0/10	12/20	0/20
1.5	0/10	0/10	10/10	0/10
2.2	2/30	0/30	10/10	0/10
3.3	18/20	0/20	10/10	8/10
5.0	30/30	20/30	—	—
†	‡		‡	
PD 50	2.7 ± 0.15 mM/kgm.		0.96 ± 0.08 mM/kgm.	
LD 50	4.6 ± 0.3 mM/kgm.		2.9 ± 0.3 mM/kgm.	

\* Dead after 24 hours.

† PD 50 = paralytic dose for half the group.

‡ Standard Deviation.

of 7.0 to 7.7. Such solutions were clear but sometimes became somewhat turbid on dilution with distilled water.

2. PHARMACOLOGICAL PROPERTIES. *Paralyzant Activity and Toxicity.* The paralyzant activity and the acute toxicity of the acid succinate of 3-(o-toloxyl)-1,2-propanediol (MAS) were determined in adult male albino mice. The drug was injected intraperitoneally into mice weighing 18 to 20 gm. which were observed thereafter at intervals for 24 hours. The mice were watched closely for the first three hours for loss of the righting reflex, which was taken as the criterion of paralysis. They were observed again at the end of 24 hours at which time mortalities were recorded. A parallel series of observations was recorded at the same time for the parent compound, Myanesin. A summary of data on the incidence of paralysis and death is presented in table 1.

MAS is approximately one-third as active a paralyzant as Myanesin. Myanesin paralyzed about 50 per cent of the experimental animals in doses of 1 mM/kgm. while similar doses of MAS did not visibly affect the animals. After some-

what larger doses of Myanesin (1.5 mM/kgm.) paralysis occurred in all of the animals. In contrast, doses of MAS as large as 2.2 mM/kgm. produced but few changes in the appearance and behavior of mice. After this dose, the animals did not move around their cages to the same extent as the controls and appeared somewhat subdued for one to two hours. When turned on their backs, the animals were able to right themselves as quickly and efficiently as the controls. Doses of 3.3 mM/kgm. of MAS produced paralysis in most of the animals.

With MAS the speed of onset as well as the duration of paralysis differed from these properties of the parent drug. Minimal effective doses of Myanesin produced a transient paralysis; the induction period was two to three minutes, as it was for all higher doses of the drug. With increasing doses of Myanesin the duration of paralysis increased. Minimal paralyzant doses of MAS, on the other hand, manifested this effect only after ten to twenty minutes; only the largest dose employed (5.0 mM/kgm.) effected paralysis with a speed comparable to that of Myanesin.

The effect of MAS by the intravenous route was also investigated. When a 10 per cent aqueous solution was injected into rabbits in doses of 100 mgm./kgm., at a rate of 100 mgm. per minute, no symptoms of any kind were observed. A similar dose injected rapidly caused paralysis of four minutes' duration which was followed by complete recovery. During paralysis, the animal appeared conscious and respiration was somewhat increased in rate and depth.

Death from Myanesin or from MAS was due to respiratory failure; the circulatory system was primarily unaffected. Neither MAS nor Myanesin possessed curare-like action. Paralysis was due, as with Myanesin, to the depressant action of the drug on the central nervous system.

*Pentamethylentetrazol (Metrazol) Antagonism.* Because of the possibility that MAS may produce pharmacological effects in non-paralyzant doses, the Metrazol antagonism was investigated. This method permitted the evaluation of effects which were not apparent when animals were given paralyzing doses.

Graded doses of Myanesin and MAS were injected intraperitoneally simultaneously with a certainly lethal dose (LD 99) of Metrazol to groups of white male mice weighing 18-20 gm. Controls which received only Metrazol were included with each experimental group. The number of mice dying at intervals of ten minutes was noted for a period of 60 minutes or longer. The results of these experiments are given in table 2.

Most of the control mice that received only Metrazol died in convulsions within ten minutes after administration of the drug. The simultaneous administration of 0.5 mM/kgm. of Myanesin protected all the animals from death for at least 30 minutes. MAS administered similarly had a much smaller protective action, the smallest dose protecting all the animals from death for at least 30 minutes being 1.5 mM/kgm. With either drug about half of the paralyzing dose had to be given in order to achieve complete protection.

The duration of action of MAS was compared with that of Myanesin. Graded doses of Myanesin or MAS were injected into groups of mice, but in these tests

TABLE 2

*The effect of various doses of Myanesin and of the acid succinate of 3-(o-toloxyl)-1,2-propanediol (MAS), respectively, in preventing death by 100 mgm./kgm. of Metrazol*

Metrazol and one of the other drugs were given simultaneously by intraperitoneal injection to white mice. In each instance, the volume of fluid injected was the same (0.8 cc./20 gm. of body weight).

METRAZOL	MYANESIN	MAS	NO. DEAD NO. USED		
			Time after injection		
			10'	30'	60'
mgm./kgm.	mM/kgm.	mM/kgm.			
100	—	—	31/35	34/35	34/35
100	0.3	—	4/10	7/10	7/10
100	0.5	—	0/10	0/10	2/10
100	0.7	—	0/15	0/15	3/15
100	1.0	—	0/10	1/10	4/10
100	1.5	—	0/20	0/20	2/20
100	2.2	—	0/5	0/5	0/5
100	—	0.7	9/10	9/10	9/10
100	—	1.0	5/15	7/15	8/15
100	—	1.5	0/20	0/20	2/20
100	—	2.2	0/5	0/5	0/5

TABLE 3

*Duration of protective effect of Myanesin and of the acid succinate of 3-(o-toloxyl)-1,2-propanediol (MAS) in preventing death from Metrazol*

Metrazol was injected 30 minutes after administration of one of the other drugs. All substances were given intraperitoneally to white mice in a volume of 0.4 cc. per 20 gm. of body weight. The controls received 0.4 cc. of distilled water instead of the depressant.

METRAZOL	MYANESIN	MAS	NO. DEAD NO. USED		
			Time after injection of Metrazol		
			10'	30'	60'
mgm./kgm.	mgm./kgm.	mgm./kgm.			
100	—	—	37/40	38/40	38/40
100	0.7	—	5/5	5/5	5/5
100	1.0	—	7/10	7/10	8/10
100	1.5	—	6/20	7/20	7/20
100	2.2	—	1/10	1/10	4/10
100	—	0.7	5/5	5/5	5/5
100	—	1.0	8/10	9/10	9/10
100	—	1.5	7/20	9/20	10/20
100	—	2.2	0/10	0/10	3/10

Metrazol was not injected until 30 minutes later. The number of deaths was again counted at ten-minute intervals for one hour or more (table 3).

The results showed that under these conditions both drugs possessed similar protective actions. The protective dose of MAS did not differ much from that observed when the drug was injected simultaneously with Metrazol. Myanesin, on the other hand, lost a considerable part of its protecting action when the injection of Metrazol was delayed for 30 minutes. While only one-half of a paralyzing dose was protective on simultaneous administration, more than twice the paralyzing dose of Myanesin was required for protection if the injection of the convulsant was delayed. With MAS as little as three-quarters of a paralyzing dose effectively protected the animals from Metrazol administered simultaneously with, or 30 minutes after, the depressant. These experiments show that MAS had an action of considerably longer duration than Myanesin.

*The Hemolytic Action.* Both Myanesin and MAS, in concentrations of 0.5 to 0.9 per cent, caused hemolysis of washed human red cells. In the presence of serum and plasma the hemolytic power of both compounds was reduced. Dilutions of a 10 per cent aqueous solution of MAS did not cause as marked "curdling" (18) of blood as observed with similar dilutions prepared from a commercially available 10 per cent solution of Myanesin. The marked protein precipitating effect of the 10 per cent Myanesin solution may be due to the action of the solvents (ethyl alcohol and propylene glycol) necessary to keep Myanesin in solution. Both Myanesin and MAS, however, have a protein precipitating action and for this reason neither drug appears suitable for intravenous administration in a concentrated solution.

*The Effect of Liver Homogenates on MAS.* Preliminary experiments indicated that mouse liver homogenates possessed the ability to split MAS into Myanesin with the liberation of acid, presumably succinic acid. Since the results *in vitro* have some bearing on the behavior of the succinate *in vivo* they are presented briefly.

In the first experiment, a well-buffered mouse liver homogenate, pH 7.7, was incubated 2.4 hours with 0.16 mM of MAS at 37.5°C. The preparation was heated briefly at the end of the incubation to coagulate protein and filtered. The filtrate was extracted with chloroform to remove neutral o-cresol derivatives and the chloroform extract evaporated. The residue was dissolved in absolute ether, washed with aqueous sodium bicarbonate to remove traces of acidic compounds and the ether evaporated. The residue remaining after this treatment was dissolved in water. The experimental sample gave the typical ultra violet absorption spectrum of Myanesin. By comparing the optical density at 270  $m\mu$  with that of known solutions of Myanesin it was calculated that 0.031 mM (20 per cent) of the Myanesin was liberated. This figure includes a correction for a small amount of absorption at 270  $m\mu$  found in the control in which MAS was incubated with a heat denatured homogenate.

In a second experiment, the liberation of acid was followed in weakly buffered mouse liver homogenates to which equi-molar amounts of various substrates were added. No change in pH with time was observed in homogenates to which



was added either succinate or Myanesin. In contrast, the addition of MAS resulted in a fairly rapid production of acid. For comparison, ethyl acetate added to mouse liver homogenate was found to split approximately 50 times faster than the succinate.

**Discussion.** The activity and potential usefulness of Myanesin and allied compounds may be evaluated in terms of the mean paralyzing and lethal doses (11, 12). From a large number of compounds, Myanesin was selected as the most worthy of clinical trial because it had the greatest paralyzing potency and the widest margin between paralyzing and lethal doses. Judged by these criteria, Myanesin acid succinate (MAS) appeared inferior to Myanesin because of the high paralyzing dose and smaller margin of safety.

Comparison of the pharmacological properties of MAS and Myanesin showed that both compounds have qualitatively similar actions. MAS differs from Myanesin by having a less intense but more persistent effect. Paralysis occurred soon after the administration of Myanesin and was of short duration, while with MAS, paralysis occurred only after administration of large doses and then only after a latent period which was several times longer than that observed with Myanesin. These observations, combined with the knowledge that liver homogenates can hydrolyze the ester linkage, indicate that MAS is broken down in the body into Myanesin and succinic acid. This breakdown occurs at a relatively slow rate and accounts for the prolonged action of MAS. The low paralyzing potency of MAS is due to the slow release of Myanesin from MAS, i.e., the low available Myanesin concentration, and the rapid oxidation and inactivation of the free Myanesin. Thus, effective blood concentrations are present only after the administration of large doses of MAS.

The low paralyzant activity of MAS did not discourage our interest in the drug since the therapeutic effects of Myanesin itself are exerted by subparalytic doses. There is the possibility that the paralyzing dose as measured experimentally is not a true index of the potential clinical value of a drug of this type.

Screening of drugs with Myanesin-like activity could best be carried out using animals with experimentally produced dystonic or hyperkinetic states more or less comparable to the clinical entities found in human patients. Unfortunately, this was not feasible. Therefore, an indirect method of evaluation, several of which have been described (12, 19), was chosen based on the observation that Myanesin and compounds with similar action are able, in small doses, to prevent death from otherwise lethal doses of Metrazol. This method permitted a quantitative comparison of the drugs.

Myanesin administered simultaneously with Metrazol provided effective protection against the stimulant. Myanesin administered 30 minutes before Metrazol had a much lesser protective power. MAS, on the other hand, had a nearly equal protective action whether injected with or before Metrazol. These results show that the duration of this action of MAS is greater than that of Myanesin.

The oral administration in man of a solution of Myanesin in clinically effective doses not infrequently causes unpleasant side effects such as dizziness and nystagmus. These transient effects have been attributed to momentarily high blood

levels. The occurrence of these side effects with MAS should be infrequent because MAS has to be split to Myanesin before activity can be observed, while at the same time the liberated Myanesin is undergoing oxidation and degradation. These reactions would tend to maintain lower and steadier blood levels of Myanesin.

Various esters of Myanesin may undergo cleavage, *in vivo*, at different rates in various species of animals. The study of other organic esters of Myanesin therefore appears warranted.

**SUMMARY.** Since it was previously shown that 3-(*o*-toloxy)-1,2-propanediol (Myanesin) is inactivated in the body by oxidation of the terminal alcohol group, the acid succinate of 3-(*o*-toloxy)-1,2-propanediol (MAS) was prepared in an effort to obtain a more soluble compound with a more persistent action.

The pharmacological effects of MAS were qualitatively similar but less intense than those produced by Myanesin. MAS was less toxic and had a considerably weaker direct paralyzing action. The duration of action of MAS was much longer than that of Myanesin. Furthermore, in doses not causing paralysis, MAS possessed a relatively greater antagonistic action to Metrazol than did Myanesin when the Metrazol was injected some time after the MAS or Myanesin.

Liver homogenates hydrolysed MAS into Myanesin and succinic acid. The experimental observations on intact animals indicated that MAS was dealt with in a similar way *in vivo*.

#### ACKNOWLEDGEMENTS

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#### REFERENCES

1. MALLINSON, F. B.: *Lancet*, 1: 98, 1947.
2. TURNBULL, T. A.: *J. Roy. Nav. Med. Serv.*, 33: 41, 1947.
3. LYALL, A.: *Glasgow Med. J.*, 28: 313, 1947.
4. WILSON, H. B., AND GORDON, H. E.: *Lancet*, 1: 367, 1948.
5. DAVIDSON, W. H. A.: *Brit. Med. J.*, 1: 544, 1948.
6. GRIFFITH, H. R., AND CULLEN, W. G.: *Anaesthesia and Analgesia*, 27: 232, 1948.
7. STEPHEN, C. R., AND CHANDY, J.: *Canad. Med. Assn. J.*, 57: 463, 1947.
8. SCHLESINGER, E. B., DREW, A. L., AND WOOD, B.: *Am. J. Med.*, 4: 365, 1948.
9. HUNTER, T. A., AND WATERFALL, J. M.: *Lancet*, 1: 366, 1948.
10. BERGER, F. M., AND SCHWARTZ, R. P.: *J. A. M. A.*, 137: 772, 1948.
11. BERGER, F. M.: *THIS JOURNAL*, 93: 470, 1948.
12. BERGER, F. M., AND BRADLEY, W.: *Brit. J. Pharmacol.*, 1: 265, 1946.
13. WYNGAARDEN, T. B., WOODS, L. A., AND SEEVERS, M. H.: *Proc. Soc. Exper. Biol. & Med.*, 66: 256, 1944.
14. TITUS, E., ULICK, S., AND RICHARDSON, A. P.: *THIS JOURNAL*, 93: 129, 1948.
15. MORRISON, T. L., SAPERSTEIN, P., WALKER, H. A., AND RICHARDSON, A. P.: *Proc. Soc. Exper. Biol. & Med.*, 68: 339, 1948.
16. RILEY, R. F., AND BERGER, F. M.: *Arch. Biochem.*, 20: 159, 1949.
17. GRAVES, E. L., ELLIOTT, T. J., AND BRADLEY, W.: *Nature*, 162: 257, 1948.
18. OGILVIE, T. A., PENFOLD, J. B., AND CLENDON, D. R. T.: *Lancet*, 1: 947, 1948.
19. BERGER, F. M.: *Brit. J. Pharmacol.*, 2: 241, 1947.

# THE SENSITIVITY OF FROG MUSCLE TO POTASSIUM AND ACETYLCHOLINE

## I. EFFECT OF CURARE AND PHYSOSTIGMINE ON THIOCYANATE: POTASSIUM SENSITIZATION

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Specific sensitization to the potassium ion was first demonstrated by Szent-Györgyi, Bacq and Goffart (1). They found that perfusion fluid collected during faradic stimulation of the hind legs of a veratrinized frog evoked a contraction of a second unstimulated veratrinized preparation. This "humoral transmission" of the contraction was shown to be due to potassium ions released during stimulation and potentiated by veratrine (2). Bacq and his associates later showed that this potentiation of the potassium ion is a distinctive feature of the action of veratrine (3-10) although it may not explain all the effects of the drug (11).

A number of unrelated substances have a similar potassium ion potentiating effect (10). Among these, thiocyanate is notable because of the intensity, regularity and easy reversibility of its sensitizing action (5-9). In the present report the specificity of the sensitization to potassium produced by thiocyanate was studied by analyzing its inter-relations with acetylcholine, curare and physostigmine.

It is well known that the muscular effect of veratrine and the sensitization to potassium produced by it are not qualitatively impeded by curare (2, 10, 11), but no quantitative results are available. It has also been reported that under certain conditions veratrine slowly sensitizes the rectus of the frog or toad to acetylcholine (3). Since the mechanism of thiocyanate:potassium sensitization has not been clarified (10), we have attempted to determine whether or not curare quantitatively affects this sensitization and also whether or not thiocyanate:acetylcholine sensitization is similar to eserization, so that these effects might be compared with the known actions of veratrine.

**METHODS.** Thirty frogs, *Rana temporaria* (Belgian) and *Rana pipiens* were used. After pithing, the rectus abdominis muscles were separated along the median line, immersed in 100 cc. Ringer's solution, and kept in the refrigerator at about 10° C. for one to five hours. Each muscle was then suspended in a 10 cc. chamber filled with oxygenated Ringer's solution at room temperature (about 23°C.). The solution was composed of the following salts dissolved in distilled water: NaCl, 0.6 per cent; KCl, 0.0075 per cent; CaCl<sub>2</sub> (anhydrous), 0.01 per cent; NaHCO<sub>3</sub>, 0.1 per cent.

The effects of the drugs were studied in two ways:

a) The length of the muscle was recorded on a kymograph by means of a counterpoised muscle lever and ink writing pen. Shortening was induced by adding various amounts of

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potassium chloride or acetylcholine to the bath.<sup>3</sup> Sensitization to potassium was produced by immersing the muscle in a Ringer's solution containing 1:2000 sodium thiocyanate for two or three minutes before adding the stimulating drug.

b) In a number of experiments a long light lever with almost negligible inertia was used. In these, the muscle shortening was determined by the lever displacement on an arbitrary scale. Stimulation was produced by replacing the plain Ringer's solution of the bath with a Ringer's solution containing the potassium chloride or acetylcholine. The degree of shortening was read on the scale after contact for four minutes with the modified Ringer's solution. For sensitization, the stimulating substance was combined with sodium thiocyanate (1:2000) and dissolved in 10 cc. Ringer's solution. Curarization was produced by a crude preparation of curare or a purified extract from *Chondodendron tomentosum* (Intocostin). For eserization the muscle was soaked for at least three hours in Ringer's solution containing 30 mgm. physostigmine salicylate per liter.

RESULTS. *I. Thiocyanate:Potassium Sensitization.* When the rectus abdominis of the frog is immersed in Ringer's solution containing thiocyanate the muscle almost immediately becomes hypersensitive to slight increases in potassium concentration. This hypersensitivity is striking after two to three minutes of contact. However, no shortening is seen with thiocyanate alone even after prolonged contact (up to more than one hour). Normally, the addition of a minimal effective quantity of potassium chloride to a non-sensitized muscle preparation produced a slow and minimal shortening. If an equivalent amount of KCl is added to the bath after the muscle has been treated with thiocyanate, the contraction is intensified at least ten- to fifteenfold and its response is almost immediate and marked (fig. 1). This contrasts with the relatively long delay observed after exposure to isotonic glucose, which also produces a marked hypersensitivity to potassium (12). The thiocyanate effect is completely reversed by repeated washings with normal Ringer's solution (fig. 1).

The apparent intensity of the sensitization by thiocyanate is soon limited by a "ceiling effect". Thus, when the initial concentration of potassium chloride is adequate to produce a marked shortening of the non-sensitized muscle, the sensitization produced by thiocyanate appears to be relatively less.

Successive sensitizations and desensitizations can be demonstrated consistently on the same muscle. Unlike veratrine, the action of thiocyanate is largely independent of the species studied. Thus, *Rana esculenta* (Hungarian frog), *Rana temporaria* and *Rana pipiens* show similar reactions to thiocyanate:potassium although their intensities may vary moderately in the different species.

*II. Action of curare on the muscular response to thiocyanate:potassium.* The following procedure was used on the rectus of *Rana pipiens* and *Rana temporaria* in a series of experiments (cf. 9):

a) In order to obtain the same initial shortening after curarization, the dose of potassium added to the bath had to be increased slightly (from 0.5 cc. to 0.6 cc. of 2 per cent KCl in the case shown in fig. 2). It is obvious that, in this supraliminal range, a very slight spontaneous change of the potassium sensitivity can

<sup>3</sup> Acetylcholine was generously supplied through the courtesy of Hoffman-La Roche, Inc.; Intocostin was supplied by E. R. Squibb & Sons; sodium thiocyanate was specially prepared by W. R. Warner & Company.

markedly decrease the reaction to the same stimulating dose. This decrease in sensitivity was constantly observed in sixteen similar experiments and has been observed by others under similar conditions (13-15). It is not seen in isolated non-curarized muscle within this time period. Our results confirm the opinion that the sensitivity of the rectus muscle to potassium is decreased by curare.

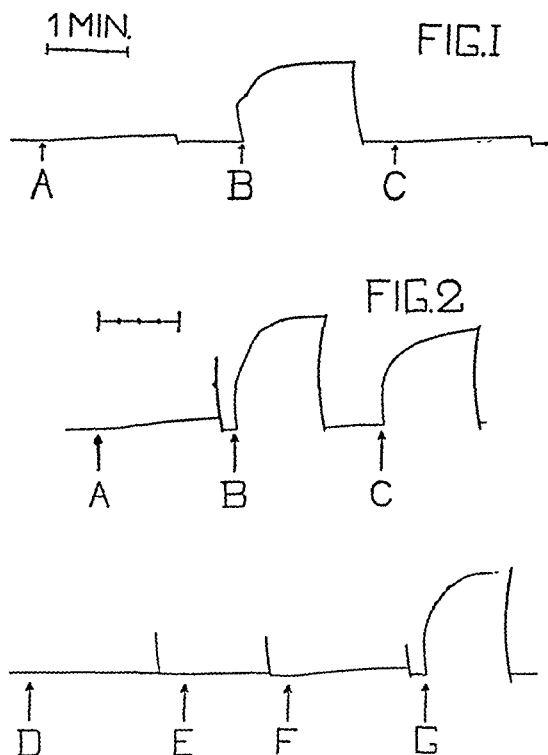


FIG. 1. MUSCLE KEPT IN RINGER'S SOLUTION FOR 75 MINUTES

Successive tests were made at intervals of fifteen minutes. A—addition of 0.6 cc. 2 per cent KCl; B—same dose of KCl, three minutes after NaSCN, 1:2000; C—same dose of KCl, after normal Ringer's solution replaced NaSCN. In this figure, as well as in the following ones, the kymograph was stopped and the effect of the drug terminated by repeated washings with normal Ringer's solution.

FIG. 2. MUSCLE KEPT IN RINGER'S SOLUTION FOR THREE HOURS

Intervals between successive tests A, B and C were fifteen minutes; between C and D, twenty minutes; between D, E and F, ten minutes; between F and G, fifteen minutes. A—addition of 0.5 cc. 2 per cent KCl; B—0.5 cc. 2 per cent KCl, three minutes after NaSCN 1:2000; C—0.5 cc. acetylcholine 100 mgm./liter; D—0.5 cc. acetylcholine 100 mgm./liter, ten minutes after Intocostrin (100 units/100 cc.); E—0.5 cc. 2 per cent KCl; F—0.6 cc. 2 per cent KCl; G—0.6 cc. 2 per cent KCl, three minutes after NaSCN 1:2000.

b) When the dose of potassium chloride has been adjusted to elicit the same initial response of the muscle, thiocyanate increases this response to the same extent whether or not the muscle has been treated by curare. The thiocyanate sensitization is therefore not affected by curarization of the preparation.

c) When the dose of potassium added to the bath is kept constant before and after curarization, there is a decrease in the response to thiocyanate:potassium

which is similar to the decrease in the reaction to potassium alone. This shows that the absolute response of the muscle to thiocyanate:potassium is largely dependent on the initial potassium sensitivity. As the latter is decreased by curare, the response to thiocyanate:potassium is similarly decreased. The process of sensitization itself is unaffected.

These conclusions were confirmed in a series of five experiments in which more quantitative data were obtained by comparing the action of several concentrations of potassium chloride, before and after sensitization by thiocyanate, on two

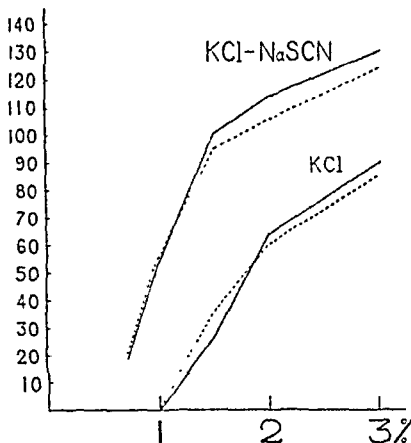


FIG. 3. COMPOSITE GRAPH OF SENSITIVITY OF FIVE NORMAL MUSCLES (SOLID LINES) AND FIVE CURARIZED MUSCLES (BROKEN LINES) TO KCl (LOWER CURVES) AND NaSCN:KCl (UPPER CURVES)

Ordinates, average shortening of the muscles, expressed in arbitrary units; abscissae, concentration in per cent of KCl solution added to potassium-free Ringer's solution (cc. KCl per 20 cc. Ringer).

matched excised recti from the same frog, one muscle immersed in Ringer's solution, the other in Ringer's solution with Intocostrin (200 to 400 units per 100 cc.).

Each muscle was first repeatedly submitted to the same concentration of potassium chloride until two successive immersions produced a similar shortening. The acetylcholine sensitivity was then roughly determined by immersion in a solution of this drug in Ringer's solution (0.05 mgm. per 10 cc. for two minutes for the normal muscle and 0.1 mgm. per 10 cc. for four minutes for the curarized muscle). The curarized muscle was completely unresponsive to this dose of acetylcholine in three instances; in the other two, some shortening occurred, but this was much less (9 per cent and 13 per cent) than that of the corresponding normal muscle.

The sensitivity to potassium was determined by exposing the control and curarized muscles to a solution containing 1 cc. of 1, 1.5, 2 or 3 per cent potassium

TABLE 1

*Effect of Intocostin on Potassium Sensitivity with and without Sodium Thiocyanate*

BEFORE TESTING	ACETYL- CHOLINE SENSI- TIVITY	NaSCN	PER CENT KCl USED (1cc. KCl/20 cc. RINGER'S SOLUTION)				
			0.75	1	1.5	2	3
Exper. 1							
a) Ringer's solution—2 hrs.	88	% 0 0.05	— 18	0 50	14 90	52 98	80 120
b) Intocostin solution—5 hrs. (200 units/100 cc.)	0	0 0.05	— 38	0 96	34 116	68 122	96 146
Exper. 2							
a) Ringer's solution—1 hr.	106	0 0.05	— 12	0 40	16 90	58 104	100 130
b) Intocostin solution—4½ hrs. (200 units/100 cc.)	14	0 0.05	— 12	0 50	38 100	76 112	110 140
Exper. 3							
a) Intocostin solution—4½ hrs. (200 units/100 cc.)	0	0 0.05	— 10	0 38	35 98	70 116	105 138
b) Ringer's solution—8 hrs.	102	0 0.05	— 24	0 66	28 132	82 148	124 172
Exper. 4							
a) Intocostin solution—4 hrs. (400 units/100 cc.)	0	0 0.05	— 18	0 36	22 54	32 62	50 76
b) Ringer's solution—7 hrs.	76	0 0.05	— 20	0 48	34 100	70 108	80 114
Exper. 5							
a) Intocostin solution—5 hrs. (300 units/100 cc.)	10	0 0.05	— 20	4 46	50 106	62 116	76 124
b) Ringer's solution—8 hrs.	92	0 0.05	— 14	0 60	44 98	58 114	78 126

Values in columns are in arbitrary units.

chloride per 20 cc. Ringer's solution. Each concentration was tested once on each muscle, the reading being taken after four minutes of contact. Between

readings the muscle was rinsed many times with normal Ringer's solution. An interval of fifteen minutes was allowed to elapse between tests. These concentrations were tested again on the same muscle in the presence of sodium thiocyanate 1:2000 added during the period of contact with potassium chloride. Because of the increased sensitivity to potassium in the presence of thiocyanate, an additional reading was taken with 1 cc. of 0.75 per cent potassium chloride per 20 cc. of Ringer's solution. During these tests the curarized muscle was immersed in Ringer's solution containing Intocostrin (300-600 units per liter).

The results may be summarized as follows (table 1):

a) Both normal and curarized muscles showed a marked increase in sensitivity to potassium in the presence of thiocyanate. As expected this potentiation was particularly marked at lower concentrations ("ceiling effect"). The minimal dose of potassium producing shortening was actually lowered by thiocyanate sensitization, thus differing from the action of veratrine (16).

b) In the first four cases the normal and curarized muscle from each pair showed significant differences in sensitivity to potassium and thiocyanate:potassium. The greater sensitivity was sometimes exhibited by the normal muscle and sometimes by the curarized muscle. No definite conclusion can be drawn from this observation since it is known that the potassium sensitivity of two homologous muscles from the same pair often shows marked differences for no obvious reason, even when the muscles are soaked in the same medium and tested simultaneously (17). For this reason the data in fig. 3 cannot be interpreted as contradicting the previous conclusion in a) above.

c) In each experiment, the difference in sensitivity to thiocyanate:potassium existing between the normal and curarized muscle closely parallels the difference in sensitivity to potassium alone (fig. 3). Data on the normal muscles (solid lines) and the curarized muscles (dotted lines) were combined in two curves of sensitivity, one for potassium and the other for thiocyanate:potassium. The superimposition of the two curves for KCl must be considered coincidental in view of the diversity of the five experiments combined in the graph, but it is significant that the displacement of the thiocyanate curve to the left is similar for the normal and the curarized muscles. This demonstrates quantitatively that curare does not affect the thiocyanate sensitization of potassium.

*III. Sensitization to acetylcholine by thiocyanate.* The mechanism of action of thiocyanate on the acetylcholine sensitivity of the normal and curarized rectus was studied in order to determine whether or not the eventual sensitization is due to physostigmine (21, 23). As far as veratrine is concerned, contradictory results have been reported concerning its effect on acetylcholine sensitivity (3, 14, 16, 18, 19, 20).

a) *Normal muscle.* The action of thiocyanate on acetylcholine sensitivity is remarkably regular and rapid. This can easily be demonstrated by comparing the effects of the same doses of acetylcholine with and without a two to three minute previous exposure to 1:2000 thiocyanate. The action of acetylcholine is consistently increased by thiocyanate although the degree of sensitization is variable from preparation to preparation. On occasion it may be slight. Under



these conditions, the response to thiocyanate:acetylcholine is consistently greater than to acetylcholine alone. Sometimes the sensitization is relatively marked, but the shortening produced by thiocyanate:acetylcholine is rarely more than double that of the control (fig. 4). In all cases, the sensitization obtained is much less marked than observed for potassium under the same conditions. It is also

FIG. 4

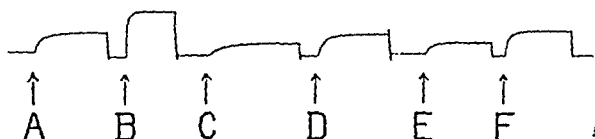


FIG. 5

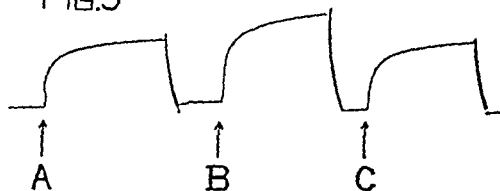
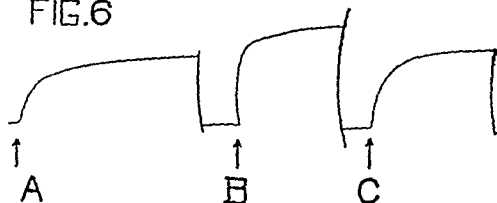


FIG. 6

FIG. 4. MUSCLE KEPT IN RINGER'S SOLUTION FOR  $3\frac{1}{2}$  HOURS

Successive tests were made fifteen minutes apart. A—0.75 cc. acetylcholine 100 mgm./liter; B—0.75 cc. acetylcholine 100 mgm./liter, three minutes after NaSCN 1:2000; C—0.5 cc. acetylcholine 100 mgm./liter; D—0.5 cc. acetylcholine 100 mgm./liter, three minutes after NaSCN 1:2000; E—0.5 cc. acetylcholine 100 mgm./liter; F—0.5 cc. acetylcholine 100 mgm./liter, three minutes after NaSCN 1:2000.

FIG. 5. MUSCLE KEPT IN RINGER'S SOLUTION FOR TWO HOURS

Successive tests were made at fifteen minute intervals. A—0.5 cc. acetylcholine 100 mgm./liter; B—0.5 cc. acetylcholine 100 mgm./liter, three minutes after NaSCN 1:2000; C—0.5 cc. acetylcholine 100 mgm./liter.

FIG. 6. MUSCLE KEPT FOR FOUR HOURS IN RINGER-PHYSOSTIGMINE SOLUTION (30 MG./LITER)

Successive tests were made at fifteen minute intervals. The muscle for this experiment was taken from the same frog as that for figure 5. A—0.25 cc. acetylcholine 100 mgm./liter; B—0.25 cc. acetylcholine 100 mgm./liter, three minutes after NaSCN 1:2000; C—0.25 cc. acetylcholine 100 mgm./liter.

immediately and completely reversible by repeated washings with normal Ringer's solution.

b) *Eserinized muscle.* Muscles were eserinized by soaking for at least three hours in Ringer's solution containing 30 mgm. physostigmine salicylate per liter. The action of physostigmine was demonstrated by the increased acetylcholine sensitivity of the preparation compared to control muscle from the same frog.

The action of thiocyanate on the acetylcholine sensitivity of eserinated muscles was similar to that seen in normal muscles. The magnitude of the sensitization was not significantly altered by physostigmine (compare fig. 5 with fig. 6). This indicates that the thiocyanate:acetylcholine sensitization is not due to inhibition of cholinesterase.

**Discussion.** The pharmacological interest in the potassium sensitization produced by thiocyanate is due to the specificity of this action. While this specificity must be considered in terms of a more complex ionic balance (3, 10), the use of the expression "sensitization to potassium" remains convenient.

The persistence of the qualitative effects of veratrine and similar drugs on curarized muscle shows that these effects, including the characteristic marked sensitization to potassium, are essentially independent of the acetylcholine which may be released by potassium. This release of acetylcholine has been demonstrated for various effectors and has also been observed in the case of the frog's rectus (17). Our quantitative studies show that there is no "acetylcholinic" role in the increased reaction to potassium produced by thiocyanate. The "veratrinic" potentiation of the potassium ion produced by thiocyanate is unaffected when curarization inhibits the effect of acetylcholine. Therefore, curare plays no part in the mechanism of the action of thiocyanate. In this sense, the action is specific for the potassium ion.

Curarization decreases the potassium sensitivity of muscle, probably as a result of a direct antagonism between curare and potassium. Curare increases the rate of loss of potassium from the muscle (22), and a decrease of the potassium content of the muscle makes it less responsive to potassium (23). Curare probably does not act through an inhibition of acetylcholine action (11).

In the present state of our knowledge, no conclusion can be drawn from the fact that physostigmine sensitizes the rectus to potassium (23) since the action of physostigmine might be complex and not simply due to inhibition of tissue cholinesterase and potentiation of the still hypothetical "acetylcholine component" of potassium action.

The concept of the duality of acetylcholine action is strengthened by our results. Physostigmine is seen not to affect the increased sensitivity to acetylcholine produced by thiocyanate. Consequently, this effect of thiocyanate is probably not due to an inhibition of cholinesterase. A direct action on the muscle is an unsatisfactory explanation since thiocyanate does not increase the shortening of the rectus when this reaction is elicited by means which do not permit the intervention of the potassium ion (*e.g.* shortening induced by quinidine (24)). Thiocyanate probably sensitizes the muscle to acetylcholine because it potentiates the effect of potassium. It is well known that acetylcholine releases potassium from sensitive preparations. Under the influence of thiocyanate this potassium would become or remain "active". The increased response of the preparation to acetylcholine would thus be due to the addition of a "potassium component", potentiated and revealed by thiocyanate, to the basic "acetylcholine component". Each component would be modified independently by the action of specific drugs.

This hypothesis also explains the remarkable disproportion between the increased responses to potassium and acetylcholine when the preparation has been exposed to sodium thiocyanate. It does not exclude the possibility that potassium may also be directly involved in the action of the "acetylcholine component" itself as a later and necessary step of this action.

#### SUMMARY

1. The properties of sodium thiocyanate as a sensitizer for the potassium ion are demonstrated by means of the frog rectus preparation. These include the effect of thiocyanate on the intensity, rapidity and reversibility of the action of potassium.

2. Curare slightly lessens the sensitivity of the preparation to potassium, but it does not quantitatively affect the process of sensitization to potassium produced by thiocyanate. Acetylcholine plays no significant part in the mechanism of the increased response to thiocyanate: potassium. The action of thiocyanate is therefore specific.

3. Thiocyanate increases somewhat the acetylcholine sensitivity of the preparation. This effect is not quantitatively affected by physostigmine.

4. The increased acetylcholine sensitivity appears to be due to the action of thiocyanate on a "potassium component" of the effect of acetylcholine.

I am greatly indebted to Dr. Louis N. Katz for editing this report.

#### REFERENCES

1. SZENT-GYÖRGYI, A., BACQ, Z. M., AND GOFFART, M.: *Nature*, **143**: 522, 1939.
2. BACQ, Z. M., AND GOFFART, M.: *Arch. Internat. Physiol.*, **49**: 189, 1939.
3. BACQ, Z. M.: *Arch. Internat. Pharmacodyn. et de Therap.*, **63**: 59, 1939.
4. BACQ, Z. M.: *Publ. della Staz. Zool. Napoli*, **18**: 1, 1939.
5. DEROUAUX, G.: *Arch. Internat. Pharmacodyn. et de Therap.*, **67**: 257, 1942.
6. BACQ, Z. M.: *Arch. Internat. Pharmacodyn. et de Therap.*, **67**: 323, 1942.
7. GODEAUX, J.: *Arch. Internat. Pharmacodyn. et de Therap.*, **67**: 425, 1942.
8. COPPEE, G.: *Arch. Internat. Physiol.*, **53**: 327, 1943.
9. VANREMOORTERE, E.: *Compt. Rend. Soc. de Biol.*, **140**: 1175, 1946.
10. BACQ, Z. M.: *Bull. Acad. Roy. Med. Belg.*, **12**: 255, 1947.
11. KRAYER, O., AND ACHESON, G. H.: *Physiol. Rev.*, **26**: 383, 1946.
12. VANREMOORTERE, E.: *Am. J. Physiol.*, **154**: 455, 1948.
13. GOUTIER, R.: Personal communication.
14. TORDA, C., AND WOLFF, H. G.: *J. Lab. Clin. Med.*, **32**: 1374, 1947.
15. KUFFLER, S. W.: *J. Neurophysiol.*, **6**: 99, 1943.
16. KUFFLER, S. W.: *J. Neurophysiol.*, **8**: 113, 1945.
17. BACQ, Z. M.: Personal communication.
18. CICARDO, V. H.: *Rev. Soc. Argent. Biol.*, **14**: 331, 1938.
19. MIYAKE, Y.: *Arb. a. d. med. Fakultat, Okayama*, **6**: 230, 1939.
20. HERRERA RAMOS, F.: *Arch. Soc. Biol. Montevideo*, **12**: 1, 1944.
21. TORDA, C., AND WOLFF, H. G.: *Am. J. Physiol.*, **145**: 419, 1946.
22. MCINTYRE, A. R., AND KING, R. E.: *Fed. Proc.*, **3**: 81, 1944.
23. TORDA, C., AND WOLFF, H. G.: *Am. J. Physiol.*, **146**: 567, 1946.
24. VANREMOORTERE, E.: Unpublished.

# ON THE MECHANISM OF THE ACUTE TOXIC ACTION OF THIOCYANATE

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The toxic manifestations of thiocyanates (SCN) have been repeatedly described in the literature (1, 2, 3), and several fatalities resulting from the therapeutic administration of the drug to hypertensive patients have been reported (4, 5). However, the mechanism of the toxic action of thiocyanates has never been satisfactorily explained. Several theories have been advanced (6, 7) but have not been confirmed by subsequent investigators (8, 9).

An experimental study on dogs was undertaken to investigate the effects of high toxic doses of thiocyanate on the oxygen content and oxygen capacity of blood. There was reason to hope that the results of such an investigation might, at least in part, clarify the mechanism of the toxic action of thiocyanate.

**EXPERIMENTAL.** Six normal male dogs, ranging in weight from 16.5 to 22 kgm., were used for the experiments. Dogs 1, 4, and 5 received toxic sublethal doses of sodium thiocyanate, dogs 2, 3, and 6 were given lethal doses. The thiocyanate was administered intravenously, as a 7 per cent sterile solution. With the exception of dog 1, which received two doses, the drug was given in a single dose. Sublethal effects were produced by the administration of 200 mgm. of NaSCN per kgm. of body weight, whereas the injection of 300 mgm. NaSCN per kgm. of body weight proved to be a fatal dose. Venous and arterial blood was drawn without stasis into heparin containing Luer lock syringes, carefully avoiding the introduction of air. The syringes were immediately capped with soldered needle ends and kept on ice until the blood was used for analysis. Samples were drawn before the injection of thiocyanate and at varying time intervals after injection. The femoral artery was punctured in most cases. The carotid artery was used in two instances. Venous blood was obtained from peripheral veins of both upper and lower limbs. The arterial and venous blood oxygen content and capacity were determined by the method of Van Slyke and Neill (10) on dogs 1, 2, 3, and 6. The method of Roughton and Scholander (11) was used for the oxygen determinations on dogs 4 and 5. The oxygen content values of venous blood samples taken from veins of both upper and lower limbs at approximately the same time, were checked on several occasions and were found to be in close agreement. Hematocrit was determined by the conventional Wintrobe method. The hematocrit readings were converted into grams of hemoglobin<sup>1</sup>, and the hemoglobin values thus obtained were used for calculating the approximate oxygen capacity. The calculated oxygen capacity values were obtained by multiplying the number of grams of hemoglobin per 100 cc. of blood by 1.34 (the amount of oxygen in cc. held by one gram of hemoglobin). Thiocyanate was determined by the method of Crandall and Anderson (12), as adapted to the Klett Summer son photoelectric colorimeter, and expressed as mgm. NaSCN per 100 cc. of plasma. All determinations were carried out in duplicate with differences between individual readings never exceeding the conventional limits of error of the methods. In all gas determinations the maximal differences between duplicate readings were within 0.2 volume per cent.

<sup>1</sup> With the aid of charts described by Phillips *et al.*, Bull. U. S. Army Med. Dept., 71, 66, 1943.

Complete control values were not obtained on dog 1. This dog fully recovered. Three weeks after the thiocyanate had been administered, no thiocyanate could be found in the plasma. At this time the blood was again analyzed for oxygen content, capacity and hemoglobin concentration. It is believed that these values can safely be accepted as controls. In dog 2 an arterial blood sample was not obtained after the injection of thiocyanate.

TABLE 1  
Complete data of one representative experiment (dog 4)

DATE AND TIME	NaSCN GIVEN	NaSCN CONC. PLASMA	HEMATO- CRIT	O <sub>2</sub> CAP. CALCUL.	O <sub>2</sub> CAP. MEASUR.	ART. O <sub>2</sub> CON- TENT	ART. O <sub>2</sub> SATU- RATION	VENOUS O <sub>2</sub> CONTENT	VENOUS O <sub>2</sub> SATU- RATION	A-V DIFFER.	REMARKS
	mgm. /kgm. body wt.	mgm. per 100 cc.		vol. per cent	vol. per cent	vol. per cent	percent	vol. per cent	percent	vol. percent	
8/24/48 9:45 AM 4:00 PM	200	0 75.4	42 40	19.0 18.1	20.3 18.05	18.5	91.0	10.1 11.25	49.7 62.3	8.4	Control Weakness, twitching of limbs and head
8/25/48 11:00 AM		68.2	41	18.5	18.07	15.2	84.1	13.62	75.4	1.58	Increasing weakness, rapid shal- low breath- ing
8/26/48 10:00 AM		49.5	37	16.75	16.41			12.61	76.8		Condition slightly im- proved, edema ankle right hind leg
8/28/48 10:00 AM		22.3	30.5	13.8	15.6	13.9	89.1	10.5	67.3	3.4	Greatly im- proved

RESULTS. The most marked change observed was a rise in the venous oxygen content and saturation. This occurred in all six dogs following the injection of thiocyanate. Dog 6 is an exception in so far as the rise in oxygen content was not accompanied by an increased oxygen saturation due to the increased oxygen capacity (probably as a result of hemoconcentration). Grossly the venous blood of the intoxicated animals assumed the bright red color of arterial blood. Simultaneously the arterial oxygen content and saturation, when determined, were found to be decreased in each instance. This simultaneous increase in the venous oxygen content and decrease in the arterial oxygen content reduced the arterio-

venous oxygen difference in four dogs studied to 1.45, 1.5, 2.0 and 1.58 vol. per cent, respectively. In the dogs that had received lethal doses of thiocyanate, a widening of the arterio-venous oxygen difference was observed in the terminal stages concomitantly with clinical signs of venous stasis, shock and circulatory collapse. \*

Significant changes in the blood oxygen capacity after the administration of thiocyanate, as compared with the assumed (calculated) oxygen capacity calculated on the basis of the hematocrit values, were not observed.

The arterial oxygen saturation after thiocyanate administration (measured on dogs 1, 4, 5 and 6) was found to be significantly reduced with a minimum reduction of 2.8 per cent and a maximum reduction of 21.9 per cent of the control saturation.

In table 1 the complete data of one representative experiment (dog 4) are given. In table 2 the means of the arterial and venous oxygen saturations and of the A-V oxygen differences at the height of the intoxication are compared with the respective control values.

TABLE 2

*Comparison of essential data before and after the administration of thiocyanate*

	MEAN CONTROL VALUES	MEAN VALUES AT HEIGHT OF INTOXICATION
Arterial O <sub>2</sub> saturation (4 dogs)—per cent .. . . .	95.7	85.1
Venous O <sub>2</sub> saturation (6 dogs)—per cent .. . . .	53.8	73.6
A-V oxygen difference (4 dogs)—vol. per cent .. . . .	6.99	1.63

Toxic manifestations were also recorded in each instance. The severity of symptoms seemed to parallel the narrowing of the arterio-venous oxygen difference, although there were wide differences in the severity of the symptoms between individual dogs. The most prominent symptom was muscular weakness. Convulsive twitching was noticed in four, diarrhea also in four, and vomiting in two dogs. Nervous symptoms, such as irritability, incoordinated movements and wild behavior were evident in four dogs, with two dogs showing the latter symptoms only slightly. Rapid, shallow breathing was observed in three animals. Dogs receiving lethal doses reached a condition of deep coma before death. In the dogs receiving sublethal doses, the severity of the clinical manifestations diminished concomitantly with widening of the oxygen span and decrease of the plasma thiocyanate concentration.

DISCUSSION. The transfer of oxygen from the blood to the tissues occurs during the blood's passage through the capillaries, and with a constant blood flow the amount of oxygen taken up by the tissues will be directly reflected by the arterio-venous oxygen difference (A-V difference). A reduced A-V difference with no change, or a decrease, in blood flow is indicative of a reduction in the oxygen uptake by the tissues. In the animals studied, the A-V differences, after the administration of high toxic doses of thiocyanate, were found to be reduced to about one-third or one-fourth of the normal resting values. The peripheral

blood flow was not measured but, on the other hand, there was nothing to suggest an increased peripheral blood flow. All blood samples were taken in a room with relatively constant temperature and while the animals were lying quietly on an animal bench. Thus it seems likely that the small A-V differences reflected an abnormally low oxygen uptake by the tissues.

The narrow A-V oxygen difference, due mainly to the rise in the venous oxygen saturation, observed with acute thiocyanate poisoning, resembles a similar reduction of the A-V difference produced by cyanide poisoning. All symptoms reported here, including gastrointestinal and nervous, can be satisfactorily explained on the basis of hypoxia (13). It seems, therefore, that the condition produced by toxic doses of thiocyanate could be regarded as one of histotoxic hypoxia (anoxia).

The high venous oxygen saturation in the intoxicated animals suggests that thiocyanate interferes with the functioning of the respiratory enzymes. This view may be supported by evidence produced by other investigators. Friend and Robinson (14) have shown that, under the experimental conditions they have used, NaSCN depressed the oxygen consumption of rat liver tissue, and they compared the depressing effects of thiocyanate to similar effects of traces of potassium cyanide and chloral hydrate. The possibility of thiocyanate being converted in the body into cyanide has been ruled out by the investigations of Taubmann *et al.* (15). The same authors also reported that the isolated muscle of the rabbit showed lowered resistance to strain and a reduction in work output when exposed to the action of thiocyanate. Barker (16) was the first to report lowered basal metabolic rates in patients receiving thiocyanate. Jung (17), starting out from the considerations, first, that all substances that have the capacity of combining with methemoglobin appear to be enzyme poisons (18), and second, that thiocyanate is known to inhibit the action of catalase, investigated and found that thiocyanate combines with methemoglobin. The formed SCN-MetHb complex was found to be less stable than any of the analogous CN—, SH—, and azide-MetHb complexes.

Direct studies of the oxygen consumption of tissue specimens, obtained from thiocyanate-poisoned animals should be performed in the Warburg apparatus in order to conclude definitely that thiocyanate interferes with normal tissue respiration. However, evidence presented supports the hypothesis that thiocyanate after entering the cells blocks the respiratory enzymes and thus interferes with the normal oxygen uptake by the cells.

The observed reductions in the arterial oxygen content and saturation were probably due to insufficient pulmonary ventilation. In the animals in which the fall in the arterial oxygen saturation was most pronounced, rapid, shallow breathing was particularly striking. The insufficient pulmonary ventilation may have been caused by a direct depression of the respiratory center. Anoxia is known to have a depressing action on the respiratory center (13, 19), but reflex stimulation from the aortic arch and the carotid bodies usually outweighs the depressing effects in the intact organism. Since stimulation of the chemoreceptors is caused by low oxygen tensions and increased H-ion concentrations, this stimulation may

will be absent in the case of the specific type of anoxia produced by thiocyanate. On the other hand, the inadequate pulmonary ventilation could have been the result of a weakness of the respiratory muscles, since, as had been stated before, general muscular weakness was an outstanding symptom.

The studies reported here are concerned only with the mode of the acute toxic action of thiocyanate. Symptoms appearing on prolonged thiocyanate therapy or experimental thiocyanate administration, such as anemia and emaciation, the goiterotrophic effects, or dermatologic manifestations (3), may depend entirely, or in part, on different mechanisms.

#### SUMMARY

1. Lethal and sublethal doses of sodium thiocyanate were given to six dogs, and changes in the arterial and venous oxygen content and capacity in peripheral blood were observed.

2. A marked rise in the venous oxygen content and saturation after thiocyanate administration was observed.

3. The arterial oxygen content and saturation after thiocyanate administration were found to be decreased significantly.

4. In four dogs on which complete studies were performed, the arterio-venous oxygen difference at the height of the intoxication was found to be reduced to an average of 1.63 vol. per cent from a control average of 6.99 vol. per cent.

5. The clinical picture of acute thiocyanate poisoning appears to be that of an histotoxic hypoxia. The hypothesis is advanced that the acute toxic effects of thiocyanates are due mainly to their respiratory enzyme blocking properties.

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#### REFERENCES

1. NICHOLS, J. B.: *Am. J. Med. Sci.*, **170**: 735, 1925.
2. GOLDRING, W., AND CHASIS, H.: *Arch. Int. Med.*, **49**: 321, 1932.
3. WALD, M. H., LINDBERG, H. A., AND BARKER, M. H.: *J. A. M. A.*, **112**: 1120, 1939.
4. RUSSELL, W. O., AND STAHL, W. C.: *J. A. M. A.*, **119**: 1177, 1942.
5. DEL SOLAR, A., DUSSAILLANT, G. G., BRODSKY, B. M., AND RODRIGUEZ, C. G.: *Arch. Int. Med.*, **75**: 241, 1945.
6. HEALY, J. C.: *New England J. Med.*, **205**: 581, 1931.
7. BANCROFT, W. D., AND RUTZLER, JR., J. E.: *J. Physical Chem.*, **35**: 1185, 3036, 1931.
8. BURKHOLDER, T. M.: *J. Lab. & Clin. Med.*, **18**: 29, 1932.
9. LINDBERG, H. A., WALD, M. H., AND BARKER, M. H.: *Am. Heart J.*, **21**: 605, 1941.
10. VAN SLYKE, D. D., AND NEILL, J. M.: *J. Biol. Chem.*, **61**: 523, 1924.
11. ROUGHTON, F. J. W., AND SCHOLANDER, P. F.: *J. Biol. Chem.*, **148**: 541, 1943.
12. CRANDALL, L. A., AND ANDERSON, M. X.: *Am. J. Dig. Dis. & Nut.*, **1**: 126, 1934.
13. VAN LIERE, E. J.: *Anoxia, Its Effects on the Body*. University of Chicago Press, Chicago, 1942.
14. FRIEND, D. G., AND ROBINSON, R. W.: *J. Lab. & Clin. Med.*, **24**: 532, 1939.



15. TAUBMANN, G. T., AND HEILBORN, R.: Arch. f. exper. Path. u. Pharmacol., 152: 250, 1930.
16. BARKER, M. H.: J. A. M. A., 106: 762, 1936.
17. JUNG, F.: Biochem. Ztschr., 304: 37, 1940.
18. AMMON-DIRSCHERL: Fermente, Vitamine und Hormone. Page 116. Leipzig, 1937. (quoted after Jung).
19. HALDANE, J. S., AND PRIESTLEY, J. G.: Respiration. Page 205. Yale University Press, New Haven, 1935.

# THE EFFECT OF PENICILLIN UPON THE CLOTTING ACTIVITY OF BLOOD IN NORMAL HUMAN SUBJECTS<sup>1</sup>

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A number of reports have appeared in the literature concerning the effect of penicillin upon the clotting activity of blood. Moldavsky, Hasselbrook, Cateno and Goodwin (1) and Macht and Ostro (2) reported that penicillin decreased the clotting time of blood in human subjects in direct proportion to the concentration of the penicillin. Macht (3) made a comparative study of the effect of the various penicillin fractions upon the clotting activity of several different species of animals (rabbit, cat and dog) and the human. The clotting activity of each was markedly decreased, more so in the rabbit than in the other species. The effect of the penicillin fractions in order of increasing activity was X, K, G, F, and G and X.

Frada (4) commented upon the "frequency of embolisms of the large blood vessels occurring in the course of penicillin therapy in acute and subacute bacterial endocarditis". He stated that penicillin increased the coagulability of the blood and diminished the resistance of the pathological segments of the endocardium with consequent formation of thrombi.

Hines and Kessler (5) studied the clotting activity of the blood of two patients with subacute bacterial endocarditis that were receiving penicillin. They reported a rise in the heparin tolerance curve, thus implying a decrease in thromboplastic activity of the blood.

Fleming (6) demonstrated that high concentrations of penicillin *in vitro* retarded blood clotting; however, the concentrations of penicillin used were much greater than the usual therapeutic level.

Because of the conflicting reports, and the widespread use of penicillin clinically today, it seemed desirable to evaluate and repeat these studies.

**PROCEDURE.** Any investigation of clotting activity of necessity involves a study of some complex phenomena concerning which our present state of knowledge is quite meager. Numerous laboratory methods for measuring the clotting activity of blood have been reported; however, each sets up a number of arbitrary conditions which cannot occur in the human body. Of all the tests devised, the simple capillary tube or the Leo White clotting method, the determination of prothrombin time, either of whole plasma as described by Quick (7), or of dilute plasma as devised by Link (8), and Shapiro (9), and the heparin tolerance curve as described by de Takats (10), probably give the most reliable results.

In the present study, the clotting activity of the blood of 24 normal subjects was investi-

<sup>1</sup> These studies were aided by a grant from the Commercial Solvents Corporation, New York New York.

gated both before and after the administration of penicillin. Capillary tube clotting times, prothrombin times by the dilute plasma method, and heparin tolerance curves were performed on three consecutive days for each subject in order to establish control levels. Three hundred thousand units of penicillin G in beeswax were then administered intramuscularly to each subject. Clotting times, prothrombin times and heparin tolerance curves were repeated one, six and twelve hours after injection.

TABLE 1

SUBJECT	CLOTING TIME BEFORE PENICILLIN DAILY CONTROLS			CLOTING TIME AFTER 300,000 UNITS PENICILLIN IN WAX		
	1	2	3	1 hr.	6 hr.	12 hr.
	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>
1	2.5	2.5	2.5	2.5	2.5	2.5
2	4.0	3.0	3.0	2.5	2.0	4.0
3	2.5	3.0	3.5	4.0	3.0	3.0
4	4.0	5.5	4.0	4.0	4.0	3.5
5	3.0	3.5	3.0	4.0	4.0	4.0
6	2.0	2.0	3.0	4.0	4.0	3.5
7	4.0	3.0	4.0	3.0	3.0	3.5
8	3.0	2.5	2.0	2.75	2.5	2.75
9	2.5	3.0	3.0	2.0	5.5	4.5
10	3.5	3.0	3.5	2.5	3.5	3.5
11	2.5	3.0	3.0	3.0	3.0	3.0
12	1.5	2.0	3.0	3.0	2.0	2.5
13	3.0	3.0	2.5	2.5	2.5	2.5
14	2.0	3.0	2.5	3.5	3.0	3.5
15	4.0	3.0	2.5	4.0	4.5	4.0
16	3.0	3.0	2.5	2.0	2.5	2.5
17	3.0	2.0	2.5	3.5	3.0	3.0
18	3.0	3.5	3.5	2.5	3.0	2.5
19	3.0	3.5	2.0	3.5	3.5	3.5
20	2.5	2.5	2.5	3.0	3.5	3.5
21	3.0	3.5	4.0	2.5	3.0	3.5
22	2.5	3.0	3.5	2.5	3.5	2.5
23	3.5	3.0	2.5	2.5	3.5	2.5
24	3.5	3.0	2.5	3.0	3.5	3.0
Daily average....	2.95	3.0	2.93	3.01	3.25	3.19
Whole average....		2.96			3.15	

The results are presented in tables 1 and 2 and figure 1. It can be seen that no significant alteration of the clotting time, prothrombin time or of the heparin tolerance curve occurred.

Four additional subjects were studied in a like manner after the injection of 300,000 units of crystalline penicillin G in saline. Again, no significant alteration of the clotting time occurred as a result of the penicillin injection (table 3).

The results of these studies indicate that penicillin fraction G caused no alteration of clotting activity in the normal human subject, when administered in the

TABLE 2

SUBJECT	PROTHROMBIN TIME BEFORE PENICILLIN	PROTHROMBIN TIME AFTER PENICILLIN		
		1 hr.	6 hr.	12 hr.
	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>	<i>seconds</i>
1	—	—	—	—
2	—	—	—	—
3	—	—	—	—
4	39.6	47.5	43.0	39.0
5	35.7	30.0	30.5	30.5
6	33.5	33.0	34.0	34.0
7	35.5	34.0	34.5	35.5
8	27.1	29.0	34.5	30.0
9	—	—	—	—
10	35.0	36.0	39.5	38.5
11	41.5	41.5	44.0	40.5
12	—	—	—	—
13	32.75	31.7	37.7	43.0
14	36.0	38.5	36.0	35.5
15	43.0	47.5	36.6	37.25
16	36.2	41.25	38.0	38.25
17	40.0	43.75	—	33.0
18	35.5	40.75	40.25	44.0
19	35.75	37.0	40.0	43.5
20	38.5	43.0	39.75	33.5
21	32.5	33.5	34.5	34.25
22	33.5	37.5	43.5	34.0
23	33.5	29.0	35.5	37.0
24	40.5	33.0	45.0	37.0

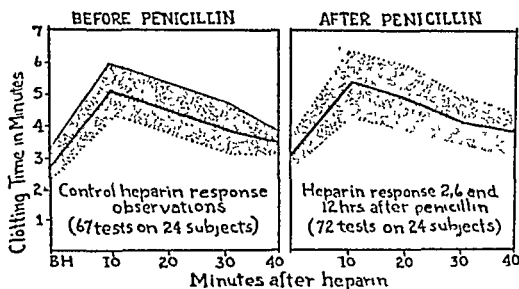


FIG. 1

usual therapeutic dosages. We think it particularly significant that these studies were made on normal human subjects rather than upon subjects with vascular diseases where emboli and thrombosis are the rule instead of the exception.

TABLE 3

SUBJECT	CLOTTING TIME BEFORE PENICILLIN DAILY CONTROLS			CLOTTING TIME AFTER 300,000 UNITS PENICILLIN IN SALINE		
	1	2	3	1 hr.	6 hr.	12 hr.
	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>	<i>minutes</i>
1	3.1	3.25	4.25	5.7	2.5	4.7
2	3.0	3.0	3.0	4.0	2.7	2.7
3	3.7	3.0	3.2	4.2	4.0	3.0
4	4.0	4.0	4.0	3.5	3.25	3.0
Daily average . . .	3.46	3.31	3.61	4.35	3.1	3.35
Whole average . . .		3.46			3.6	

## SUMMARY

1. The effect of penicillin on the clotting activity of blood was measured in 24 normal subjects before and after the administration of 300,000 units of crystalline penicillin G in wax and oil. There was no significant alteration in the clotting time, prothrombin time, or heparin tolerance curve.

2. Similar results were obtained with four normal subjects following the administration of 300,000 units of crystalline penicillin G in saline.

3. It is concluded that penicillin G has no effect on the clotting activity of blood in normal human subjects.

## REFERENCES

1. MOLDAVSKY, L. F., HASSELBROOK, W. B., AND CATENO, G. D.: *Science*, **102**: 38, 1945.
2. MACHT, D. I., AND OSTRO, M.: *Science*, **103**: 402, 1946.
3. MACHT, D. I.: *Science*, **105**: 313, 1947.
4. FRADA, G.: *Giorn. Med. Palermo*, **3**: 95, 1946.
5. HINES, L. E., AND KESSLER, D. L.: *J. A. M. A.*, **128**: 794, 1945.
6. FLEMING, A., AND FISH, E. W.: *Brit. Med. J.*, Aug. 16, 1947, p. 242.
7. QUICK, A. J.: *J. A. M. A.*, **110**: 1658, 1938.
8. CAMPBELL, H. A., SMITH, W. K., ROBERTS, W. L., AND LINE, K. P.: *J. Biol. Chem.*, **138**: 1, 1941.
9. SHAPIRO, S., SHERWIN, B., REDISH, M., AND CAMPBELL, H. A.: *Proc. Soc. Exper. Biol. and Med.*, **50**: 85, 1942.
10. DE TAKATS, G.: *Surg. Gyn. and Obstet.*, **77**: 31, 1943.

# ON THE ANTAGONISM BETWEEN PRESSOR AND DEPRESSOR AGENTS IN THE FROG'S HEART<sup>1</sup>

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It has recently been shown that tetraethylammonium (TEA), known to antagonize the action of vagomimetic drugs on the frog's heart, antagonizes also the action of potassium (K) (1). In order to find a unitarian explanation of this result, the possibility was discussed whether the final effect of vagomimetic drugs was due to K liberated by them. It has been definitely proven that K can liberate acetylcholine (AcCh) (2) but not the reverse, i.e. that AcCh can liberate K. If the latter were so it is to be expected that any pressor drug which antagonizes the effect of K should also influence that of AcCh. The question appeared to merit further investigation.

**METHODS.** The experiments were performed on the isolated frog heart (*Rana Pipiens*) attached to a Straub cannula. The Ringer solution used had the following composition: 0.5 gm. NaCl, 0.01 gm. KCl, 0.01-0.02 gm.  $\text{CaCl}_2$ , 0.02 gm.  $\text{NaHCO}_3$  and water to make 100 cc. of solution. AcCh served as representative of the vagomimetic drugs and epinephrine and sodium oleate as pressor agents. The last two drugs are supposed to act on surface receptors other than those acted upon by AcCh or K, respectively. Hence an incidental functional antagonism would not be the consequence of competition for the same site but of the opposite functional effects (physiological antagonism). For reasons to be discussed later the action of nucleotides and nucleosides was also investigated and that of TEA re-examined.

In order to be able to compare exactly the effect of augmentor agents on the action of the AcCh and K, respectively, it was necessary to use concentrations of these depressor agents which decreased the amplitude of the ventricular beats to about the same amount. Depressor and pressor agents were not administered simultaneously; the depressor was added first and the pressor given only when the depressor effect had been fully developed.

## EFFECT OF PRESSOR AGENTS ON THE DEPRESSOR EFFECT OF AcCh AND K

**1. Epinephrine.** The comparison of the effect of epinephrine upon the ventricular amplitude previously depressed by AcCh or K to the same extent shows as a rule that during K depression epinephrine acts as it does on the normal heart whereas it is almost or entirely inactive during AcCh depression even in doses producing a maximal effect upon a normal heart (fig. 1). Fig. 2 demonstrates that epinephrine during AcCh depression has an inconspicuous effect in a dosage eight times as large as that producing a normal effect during K depression. Fig. 3 shows that an AcCh concentration which only slightly decreases the amplitude strongly inhibits the epinephrine effect.

**2. Sodium Oleate.** Upon prolonged perfusion with Ringer solution, frog hearts

<sup>1</sup> Aided by grants from the Dazian Foundation and the Rockefeller Foundation.

in time become extremely hypodynamic because of their increasing susceptibility to the effect of the K contained in the Ringer solution. This is due to the gradual loss from the heart of lipoids. Addition to the Ringer solution of these lipoids almost immediately causes the hypodynamic heart to recover (3). The lipoids

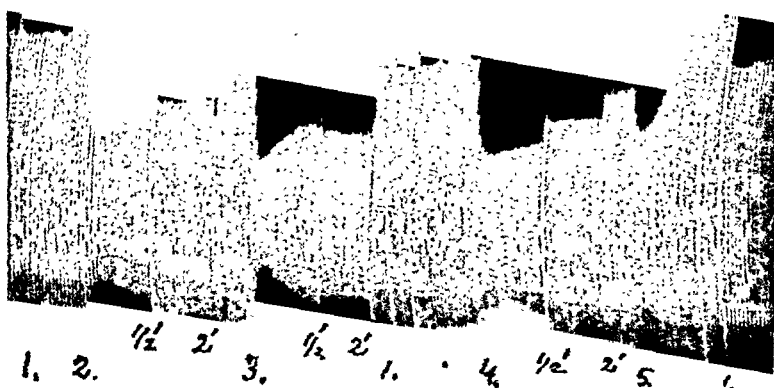


FIG. 1. INFLUENCE OF ACETYLCHOLINE (AcCh) AND POTASSIUM (K) RESPECTIVELY ON THE EPINEPHRINE EFFECT

1. Ringer; 2. AcCh  $10^{-8}$ ; 3. AcCh  $10^{-8}$  + epinephrine  $10^{-8}$ ; 4. KCl 0.05%; 5. KCl 0.05% + epinephrine  $10^{-8}$ .

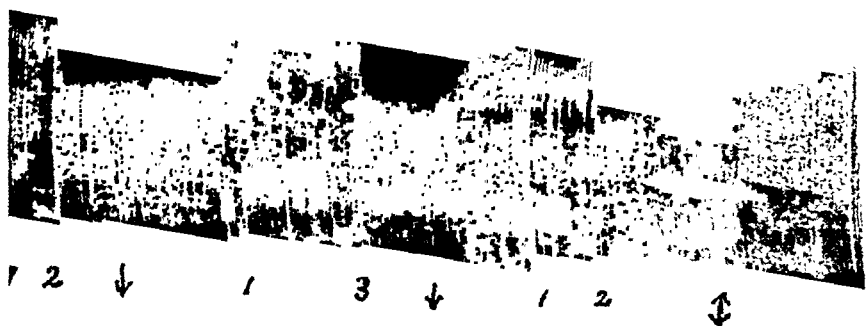


FIG. 2. INFLUENCE OF ACETYLCHOLINE (AcCh) ON THE EFFECT OF EXCESSIVE DOSES OF EPINEPHRINE

1. Ringer; 2. AcCh  $0.5 \times 10^{-7}$ ; 3. 0.66% KCl ↓ + epinephrine  $0.5 \times 10^{-8}$  ↑ + epinephrine  $4 \times 10^{-8}$ .

can be substituted by the sodium salts of some fatty acids especially of oleic acid. It has also been shown that these lipoids protect the heart against the effect of other depressants (4).

The results obtained with sodium oleate in regard to its antagonism against AcCh and K correspond with those obtained with epinephrine: oleate is without

effect against AcCh in doses where it is fully effective against K (fig. 4). Fig. 4 shows in addition that after washing out the heart by replacing the AcCh and sodium oleate-containing Ringer solution by plain Ringer solution, the full action



FIG. 3. INFLUENCE OF ALMOST INEFFECTIVE DOSES OF ACETYLCHOLINE ON THE EPINEPHRINE EFFECT

1 Ringer; 2 AcCh  $10^{-8}$ ; 3 AcCh  $0.5 \times 10^{-8}$ ; 4 + epinephrine  $0.5 \times 10^{-8}$



FIG. 4. INFLUENCE OF ACETYLCHOLINE AND POTASSIUM ON THE OLEATE EFFECT

1 Ringer, 2 AcCh  $10^{-8}$ , 3 AcCh  $10^{-8}$  + sodium oleate  $0.5 \times 10^{-8}$ , 4 KCl 0.04%, 5 KCl 0.04% + sodium oleate  $0.5 \times 10^{-8}$

of sodium oleate becomes immediately manifest. This proves that its inactivity during AcCh depression is not due to interference of AcCh with the attachment of oleate at the heart. There is no need to assume that it is different with epinephrine.



3. *Tetraethylammonium*. As shown in a former paper (1) TEA even in doses ineffective on the normal heart antagonizes the effect of vagomimetic agents and of K. Further experiments with TEA were conducted in the same way as those reported above in order to compare more exactly the effect of TEA upon the action of AcCh and K.

The experiments demonstrated, as was to be expected from the results of previously published work (1), that TEA, in contrast to epinephrine and oleate, antagonizes the action of AcCh as well as that of K (fig. 5). In addition, however, they showed (fig. 5) that the character of the recovery by TEA from depression is quite different. Though in both cases the recovery starts immediately in the case of K depression it proceeds gradually while in the case of AcCh depression the pulse amplitude rises steeply immediately after addition of TEA and usually after a few pulses the level of the normal heart beat is reached again. Not even with atropine does such a sudden rise occur. With concentrations of TEA not sufficient to produce a full recovery the rise as a rule is also steep.

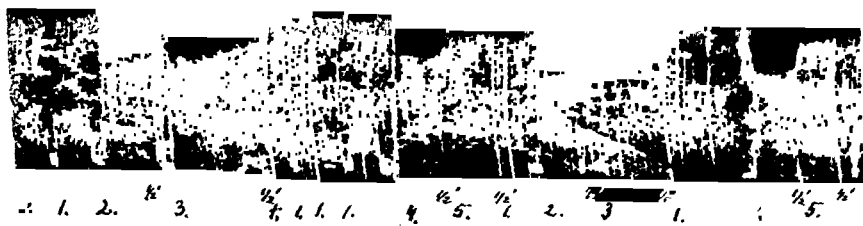


FIG. 5. INFLUENCE OF TETRAETHYLAMMONIUM (TEA) ON THE EFFECT OF ACETYLCHOLINE AND POTASSIUM RESPECTIVELY

1. Ringer; 2. 0.06% KCl; 3. 0.06% KCl + TEA  $0.5 \times 10^{-3}$ ; 4. AcCh  $0.5 \times 10^{-3}$ ; 5. + TEA  $0.5 \times 10^{-3}$ .

Concentrations of TEA which are sufficient to produce recovery from AcCh and K depression are, as has already been shown in a former paper (1), below those necessary to cause an increase of the amplitude of the beat of a normal heart. This suggests that under the condition of AcCh and K depression the heart is more responsive to TEA. Increased responsiveness, however, does not explain the difference existing between the restorative effect of TEA on hearts depressed by AcCh or K, respectively. TEA applied in doses insufficient to raise the pulse amplitude of the normal heart increases the amplitude above this level if administered during K depression but not during AcCh depression. Furthermore increased responsiveness does not explain the differences in regard to speed and character of the response to TEA by a heart depressed by AcCh or by K or by a normal heart. In the latter case, except with very high doses of TEA, the increase of the amplitude proceeds very slowly and reaches its maximum only after many minutes. Fig. 6 demonstrates this action of TEA on the normal heart and contrasts it with the action of the same dose of TEA upon the same heart depressed by a very large dose of AcCh. In view of the structural similarity between AcCh and TEA Clark (5) assumed substrate competition to be the cause

of the antagonism. As one way of checking the correctness of this view, we studied the behavior of TEA in counteracting not only the decrease of the pulse amplitude but also other characteristics of AcCh action. One of them consists, as has been shown above, in the decrease of reaction of the AcCh-depressed heart to augmentor agents such as epinephrine or sodium oleate. Hence it was investigated whether or not this unresponsiveness was counteracted by TEA. As

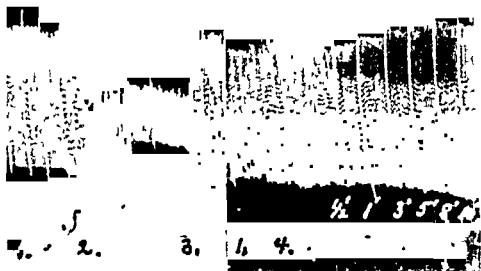


FIG. 6 COMPARISON OF THE EFFECT OF TEA ON THE NORMAL HEART AND THE HEART DEPRESSED BY AcCh

1 Ringer, 2 AcCh  $4 \times 10^{-4}$ , 3 TEA  $10^{-3}$ , 4 Ringer + TEA  $10^{-3}$



FIG. 7 CANCELLATION BY A SMALL DOSE OF TEA OF THE INEFFECTIVENESS OF EPINEPHRINE DURING ACETYLCHOLINE DEPRESSION

1 Ringer, 2 AcCh  $10^{-4}$ , 3 + epinephrine  $10^{-4}$ , 4 + TEA  $10^{-4}$

fig. 7 shows this is actually the case: epinephrine, without effect if added during AcCh depression, becomes fully effective if added even during a slight recovery by TEA from AcCh depression. This result proves that TEA acts by substrate competition

The possibility originally considered as to whether the final effect of AcCh might be due to release of K is definitely excluded by the sole fact that the reaction to pressor agents of a heart depressed by AcCh or K, respectively, is quite different

As to the cause of the inhibition of the effect of the pressor agents, except that of TEA by AcCh, the fact that an inconspicuous diminution of the heartbeats produced by AcCh is accompanied by a great unresponsiveness to the effect of pressor agents and that this unresponsiveness is even absent during a deep depression of the beats produced by K in the same heart indicates that diminution of contractility as such is not the cause.

It is most likely that the specific inhibitory state produced by AcCh is caused by its influence upon a unitarian underlying mechanism needed to maintain the various physiological functions such as frequency, conduction, contractility and excitability in a normal state. Starting from this view Garrey and Boykin (6) investigated whether vagus stimulation would affect metabolism. They found that the  $O_2$  consumption of the resting frog's auricle was deeply depressed by vagus stimulation whereas K even in excessive concentration (fifteen times the



FIG. 8. INFLUENCE OF ACETYLCHOLINE IF ADMINISTERED PREVIOUSLY ON THE EFFECT OF ADENOSINETRIPHOSPHATE (ATP)

1. Ringer; 2. ATP  $0.25 \times 10^{-5}$  M; 3. AcCh  $10^{-8}$ ; 4. AcCh  $10^{-8}$  + ATP  $0.25 \times 10^{-5}$  M.

normal) (7) had very little or no effect (6). They found, furthermore (8), that HCN decreased the  $O_2$  consumption to the same extent or even more than vagus stimulation without, however, producing the symptoms characteristic of vagus inhibition. Hence Garrey (6) correctly concluded that the decrease of the  $O_2$  consumption by vagus stimulation is not the cause of the inhibitory actions but simply accompanies them.

EFFECT OF NUCLEOTIDES AND NUCLEOSIDES ON THE DEPRESSOR EFFECTS OF AcCh AND K. In view of the great speed with which the inhibitory state is established by vagus stimulation or AcCh it is necessary to consider (see for example (9)) whether there is interference in some way with a process responsible for the immediate supply of energy needed for all functions. From this point of view it seemed worthwhile to investigate whether or not AcCh would influence the effect of adenosinetriphosphate (ATP) whose breakdown is supposed to be nearest, in time at least, to contraction. We conducted such experiments, though on the one hand analyses of Clark and Eggleton (10) provided no evidence

that ATP supplies energy to the contraction process of the frog heart, and on the other hand the visible effect of ATP on the heart's activity cannot be unrestrictedly ascribed to its metabolic functions.

As far back as 1930 Lindner and Rigler (11) showed that ATP in a concentration as low as  $10^{-7}$  produced an immediate and steep transitory rise of the amplitudes of the frog heart followed by a much slower second rise. Applying ATP sodium prepared from barium or calcium salts, obtained from different sources, we were able to duplicate exactly the results of Lindner and Rigler employing their concentrations (fig. 8 and 9). We observed furthermore that after a certain time, the duration of which depended on the dose applied, the amplitude returned slowly to the previous normal level. However after pretreatment with very small concentrations of AcCh the addition of ATP in concentrations varying from  $10^{-6}$  to the strongly effective level of  $0.5 \times 10^{-5}$  still produced the primary

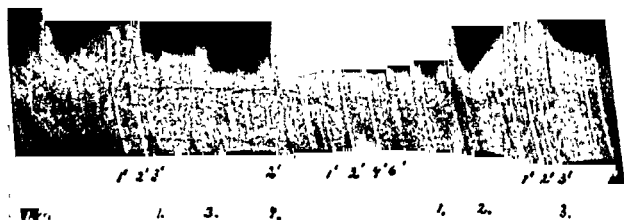


FIG. 9. INFLUENCE OF ACETYLCHOLINE IF ADMINISTERED PREVIOUSLY OR SUBSEQUENTLY ON THE EFFECT OF ADENOSINETRIPHOSPHATE (ATP)  
1. Ringer; 2. ATP  $0.25 \times 10^{-5}$  M; 3. AcCh  $10^{-6}$ ; 4. AcCh  $10^{-6}$  + ATP  $0.25 \times 10^{-5}$  M.

rise but the slow secondary rise was entirely or almost entirely inhibited (fig. 8 and 9) exactly as was the case with epinephrine and oleate. As mentioned above the effect of ATP fades off after a while. The Ringer solution withdrawn at this time and tested for ATP activity by administration to a normally beating heart proved to be without effect, thus indicating that ATP was no longer present. ATP was found to disappear in the same length of time when it had been administered to a heart previously depressed by AcCh.<sup>2</sup> Furthermore the effect of corresponding concentrations of the sodium salts of ADP, muscle AMP, adenosine and pyrophosphate was tested both on the normal heart and after previous administration of AcCh: ADP and AMP acted exactly as did ATP. Adenosine did not produce the primary rise and the increase of the amplitude was much slower and smaller; furthermore the increase in response with increasing concentrations was strikingly small. In all cases AcCh inhibited the increase, except

<sup>2</sup> In this case the test for ATP was carried out after previous atropinization of the test heart.

for the primary rise, and did not interfere with the gradual inactivation of these compounds as judged by the test employed.

As with epinephrine and oleate the inhibition by AcCh of the pressor effects of the compounds just discussed is specific since it is not produced by K.

The results showing that the compounds vanish from the fluid exactly at the same moment, independent of whether or not the heart was previously influenced by AcCh, indicates that AcCh does not interfere with their observable fate. The results, however, do not permit a decision as to whether the gradual increase of the heartbeats produced by these compounds is due to their specific metabolic properties or whether the compounds act as whole molecules. Hence we cannot decide either whether the suppression by AcCh of their augmentor action is due to inhibition of a metabolic activity or of responsiveness to their augmentor action as in the case of epinephrine and oleate. We consider the second alternative to be much more likely as many pharmacological effects of these agents have been revealed which cannot possibly be due to the wellknown metabolic actions of ATP *in vitro* (13).

DISCUSSION. For reasons mentioned in the introduction we compared the response to pressor agents of hearts depressed by AcCh or K, respectively. We found that the response during AcCh depression was extremely poor whereas it was not different from that of a normal heart during K depression. Decrease of responsiveness to stimulants as a syndrome of a specific inhibitory state is produced not only by AcCh but also by epinephrine in organs inhibited by it. More than half a century ago Jacobj (14) obtained a vagus effect on the intestine of starved rabbits only after previous severing of the inhibitory splanchnic nerves. Elliott (15) was unable to elicit the pressor effect of pelvic stimulation of the bladder during epinephrine inhibition. Bozler (16) found under the same conditions a diminished response of the intestine and the uterus even to electrical stimulation. The well known fact that during epinephrine inhibition the automatic movements of the bladder and of the intestine can completely cease demonstrates that the effect of stimuli from within can also be decreased or entirely prevented.

We do not yet know what are the intermediary steps leading to the final effects of AcCh or epinephrine administration but we have some clues as to what may be the first stage. It is widely accepted that AcCh and epinephrine first react with specific receptors at the cell membrane and thereby influence the polarized state of the membrane. Where the change consists in depolarization such as that produced by AcCh at the motor endplate, impulses are set up. In this connection it should be mentioned that as far back as 1887 Gaskell (17) demonstrated that stimulation of the accelerator nerve produced a negative potential in the resting auricle of turtle hearts. On the other hand he found that vagus stimulation of the same preparation increased polarization. The result was repeatedly confirmed (Samojloff (18), Meek and Eyster (19), Bayliss (20), and Garrey (21)). Later on Bacq and Monnier (22) and Dubuisson and Monnier (23) claimed that not only in the heart but in all organs the inhibitory action of both epinephrine and AcCh was preceded by an increase and the augmentory

action by a decrease of polarization. The view that AcCh might act by altering the electrical behavior of the cell membrane has been offered even by textbooks (21).

K like AcCh stimulates the striated muscle—K by depolarizing in every region, AcCh by depolarizing only the motor endplate (25)—and K abruptly decreases the amplitude of the heartbeats exactly like AcCh. One could therefore be tempted to assume that the action of K here too might be due to increased polarization as is surmised for AcCh.<sup>2</sup> Such an assumption however, if unqualified, would not take into account the fact that there exist differences between the effects of AcCh and K on the heart as evidenced by the fact that only AcCh leads to decrease of O<sub>2</sub> consumption and of responsiveness to pressor agents and to abbreviation of the refractory period (26). In addition we have to stress the following point: whereas it is certain that the depolarization produced by AcCh and K sets up impulses within the striated muscle it is not definitely proven whether the increased polarization provoked by AcCh within the heart is responsible for all the inhibitory effects. This could be decided only by determining whether or not the effect of increased polarization brought forth by an electrotonus of an atropinized heart would mimic all the effects of AcCh. We refrain from discussing other possibilities as to the mode of action of AcCh and K as we are not able to present working hypotheses.

#### SUMMARY

1) During depression of the amplitude of the heartbeats by acetylcholine but not by potassium the responsiveness of the heart to the augmentory action of epinephrine and sodium oleate is greatly diminished.

2) The depressor action of acetylcholine and potassium is antagonized by tetraethylammonium. In the case of acetylcholine this antagonism is due to substrate competition.

3) In the normal and in the potassium depressed heart, ATP, ADP and AMP produce an immediate and steep transitory increase of the amplitude of the heartbeats, followed by a gradual rise which slowly fades. The second increase is suppressed during acetylcholine depression.

4) The mechanism involved in these actions is discussed.

#### REFERENCES

- 1 LOEWI, O · *THIS JOURNAL*, 88: 136, 1946
- 2 BROWN, G L, AND FELDBERG, W · *J. Physiol.*, 86: 200, 1936, FELDBERG, W, AND GUIMARIS, T. A · *J. Physiol.*, 86: 306, 1936
- 3 CLARK, A. J · *J. Physiol.*, 47: 66, 1913
- 4 LOEWI, O · *Pflüger's Arch.*, 187: 123, 1920
- 5 CLARK, A. J, AND RAYENOS, T · *Quart. J. Exper. Physiol.*, 26: 375, 1937.
- 6 GARREY, W D, AND BORKINS, T T · *Am. J. Physiol.*, 109: 286, 1934
- 7 GARREY, W D · Personal communication, 1948
- 8 GARREY, W D, AND BORKINS, T T · *Am. J. Physiol.*, 111: 640, 1935

<sup>2</sup> The effect of K on the state of polarization within the heart has to our knowledge not been investigated

9. CLARK, A. J.: Metabolism of frog heart, p. 208, 1938.
10. CLARK, A. J., AND EGGLETON, M. G.: Quart. J. Exper. Physiol., **26**: 119, 1936.
11. LINDNER, F., AND RIGLER, R.: Pflueger's Arch., **226**: 697, 1930.
12. BUCHTHAL, F., AND KAHLSON, G.: Acta Physiol. Scand., **11**: 284, 1946.
13. FELDBERG, W., AND HEBB, C.: J. Physiol., **107**: 210, 1948; EMMELIN, N., AND FELDBERG, W.: Brit. J. Pharmacol., **5**: 273, 1948.
14. JACOB, C.: Arch. Exper. Pathol. and Pharmacol., **29**: 171, 1893.
15. ELLIOTT, T. R.: J. Physiol., **32**: 401, 1905.
16. BOZLER, E.: Am. J. Physiol., **130**: 627, 1940.
17. GASKELL, W. H.: J. Physiol., **8**: 404, 1887.
18. SAMOJLOFF, A.: Centralbl. Physiol., **27**: 575, 1913; Pflueger's Arch., **199**: 579, 1923.
19. MEEK, W. I., AND EYSTER, T. A. E.: Am. J. Physiol., **30**: 271, 1912.
20. BAYLISS, W. M.: Principles Gen. Physiol., 1920, p. 407.
21. GARREY, W. E.: Am. J. Physiol., **68**: 128, 1924.
22. BACQ, Z., AND MONNIER, A. M.: Arch. Internat. Physiol., **40**: 467, 1935.
23. DUBUISSON, A., AND MONNIER, A. M.: Arch. Internat. Physiol., **38**: 207, 1934.
24. Howell's Textbook of Physiology, p. 787, 1946
25. KUFFLER, S.: J. Neurophysiol., **6**: 99, 1943
26. KANDA, Z.: Arch. f. Exper. Pathol., u. Pharmacol. **190**: 417, 1938

# EXPERIMENTAL CHEMOTHERAPY OF TRYPANOSOMIASIS

## I. EFFECT OF p-PHENYLENE DIGUANIDINE AND RELATED COMPOUNDS AGAINST EXPERIMENTAL INFECTIONS WITH *Trypanosoma equiperdum*

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### INTRODUCTION

A search was begun in our laboratories in 1944 for a compound which might possess advantages over existing agents for the treatment of trypanosomiasis, and particularly for one which would be effective when administered orally. Very early in the course of this screening program it was found that p-phenylene diguanidine possessed curative properties against *Trypanosoma equiperdum* in mice when administered either orally or intraperitoneally. It was not as powerful a trypanocide as Bayer 205 or several other known compounds, but it possessed several interesting properties which encouraged extensive investigations. The parasitological data obtained with p-phenylene diguanidine and several related compounds in experimental animals is presented in this paper, and the chemistry of the group is discussed in detail elsewhere (1).

The effectiveness of guanidine compounds in experimental trypanosomiasis was first demonstrated by British investigators. King, Lourie and Yorke (2) showed that a series of homologues of Synthalin possessed trypanocidal properties. The trypanocidal action rose as the number of methylene groups in the alkyl chain was increased; the decane, dodecane and tetradecane derivatives were the most active. Activity was then found in alkylene diamidines in which two guanyl groups were attached to the ends of the methylene chain without the intervention of other groups. Lourie and Yorke (3) then proceeded to aromatic diamidines and selected 4,4'-diamidino stilbene (Stilbamidine) as the most active member of the group. St. A. Heathcote (4) gives a recent summary of the pharmacology and therapeutic uses of Stilbamidine and related compounds.

The guanidine compounds described in the present report, and more particularly p-phenylene diguanidine, are not so strikingly effective in laboratory infections with trypanosomes as are the aromatic diamidines. p-Phenylene diguanidine, however, has a relatively simple structure, and it cures *T. equiperdum* infections in mice in well-tolerated doses when administered orally.

**MATERIAL AND METHODS.** Mice infected experimentally with *T. equiperdum* were used as laboratory hosts throughout the major part of these investigations. Each mouse was inoculated intraperitoneally with a saline suspension containing approximately 50,000 to 100,000 trypanosomes, or an average of 2.5 million trypanosomes per kilogram body weight in 20 gram mice. This parasite produces highly consistent infections in untreated mice, with death occurring almost invariably within three to five days after inoculation with the above



number of trypanosomes. The strain of *T. equiperdum* used was obtained in 1945 through the kindness of Dr. A. L. Tatum, University of Wisconsin.

Some deviations from the traditional testing methods for trypanocides used by many investigators have been used in our program. Most screening tests performed with arsenicals have involved the "single dose technique"; that is, one dose of the drug is administered 24 hours after inoculation with parasites. Minimum therapeutic doses and curative doses are then determined according to the number of animals which survive over a thirty-day period. The single dose which kills one-half of the animals ( $LD_{50}$ ) divided by the single dose which cures one-half of the animals ( $CD_{50}$ ) is called the chemotherapeutic index. This is a well recognized standard and is used in a number of other *in vivo* biological assays.

The single dose technique appears to provide a satisfactory trypanocidal assay with compounds which are not excreted rapidly. For screening new compounds about which nothing is known concerning absorption and excretion, however, the exclusive use of this method could conceivably bypass many types of compounds which might possess trypanocidal properties when administered more frequently. In our screening program, therefore, multiple doses of compounds were administered to infected mice over a period of at least three days after inoculation with parasites. The variations of the technique used are described below. For routine screening, compounds soluble in water were administered intraperitoneally in most cases, and compounds insoluble in water were administered orally in a 2.5 per cent suspension of corn starch. This was purely a matter of convenience in the initial screening tests, since in our hands the intraperitoneal method of administration when possible was easier, faster and less liable to produce death by mechanical injury in mice than oral administration. If sufficient compound was available, 50 mgm. per kgm. intraperitoneally, or 200 mgm. per kgm. orally was used as the initial dosage for the first screening test. If death from toxicity occurred at these doses the quantity was halved and tested again. Active compounds were retested at several dosage ranges.

*Method A.* For preliminary assays of all new compounds, dosage was administered once on the day of inoculation with parasites, and twice daily thereafter with eight hours between doses, for three days. Untreated controls were always included. Since *T. equiperdum* invariably kills mice on the third to fifth days after inoculation, trypanocidal or suppressive effects could be determined one or two days after treatment was stopped, without necessitating counting the numbers of trypanosomes present. This procedure provides a quick method for spotting active substances, and is also valuable for comparing the action of different derivatives within any group of compounds. In screening tests mice were not held for extended observation periods after cessation of treatment.

*Method B.* After preliminary tests had shown that p-phenylene diguanidine appeared to be the most promising of any of the guanidine derivatives tested, several series of mice were treated twice or three times daily for from 3½ days to 2 weeks after inoculation with parasites, in order to determine whether the curative dose could be lowered by repeated dosage. The first group of mice listed in table 2 were treated once on the day of inoculation with trypanosomes, 3 times daily for the next 4 days, with 4-hour intervals between dosage, twice on the 6th day, and once on the 7th day. The second group were treated similarly for the first week, and were then treated 3 times daily from the 8th to the 12th days, with 2 doses on the 13th day and 1 dose on the 14th day. The third group were treated once on the 1st and 7th days, and twice on the 2nd to 6th days with 8-hour intervals between doses. The fourth group was treated once on the 1st, 7th and 14th days, and twice daily on the 2nd to 6th days and the 8th to 13th days. These mice were then observed for 30 days after cessation of treatment to determine curative effects. Mice which lived throughout the period of observation were considered cured. It was assumed that the mice which died during this period succumbed to their trypanosomes, even though parasite counts were not made immediately before death. This procedure has been used by many investigators previously, and has become a more or less standard practice in the experimental chemotherapy of *T. equiperdum* infections. The comparison between the activity of several guanidines, Stilbamidine, and Bayer 205 given in table 7 were made on the basis of multiple doses with all compounds listed.

*Method C.* In order to test the efficacy of p-phenylene diguanidine against *T. equiperdum* after the parasites had had an opportunity to become well established in the host, one series of mice was not treated until 54 hours after inoculation with parasites. These data are given in table 9.

Several different samples of p-phenylene diguanidine were used for bioassay. The first two batches received were impure bicarbonate salts. These were tested as such, and the second batch was then purified. The hydrochloride was prepared from the second batch, recrystallized from aqueous alcohol. A third batch was received as the mixed carbonate and bicarbonate salts. Unless otherwise noted, the results of tests given in the tabular data were obtained with the hydrochloride.

**DEFINITION OF TERMS.** The following definition of terms is given as they are used in the present paper:

*Suppressive effect.* Treated mice lived for varying periods of time after all controls were dead, but less than 50 per cent survived the 30-day observation period after cessation of treatment.

*Curative effect.* Fifty per cent or more treated mice survived for 30 days after cessation of treatment.

*Minimum therapeutic dose (M.Th.D.).* The least dose needed to produce a suppressive effect.

*Curative dose (CD<sub>50</sub>).* The least dose needed to produce a curative effect.

*Lethal dose (LD<sub>50</sub>).* The least dose needed to kill 50 per cent or more mice within 21 hours after a single dose, or on the first or second day of multiple dosage when multiple dosage was used.

*Chemotherapeutic Index (C.I.).* The lethal dose divided by the curative dose. When multiple dosage was used the chemotherapeutic index was determined on this basis.

**RESULTS.** *p-Phenylene Diguanidine.* The oral and intraperitoneal curative doses of this compound in mice are listed for 21 series of tests in table 3. Tables 1 and 2 illustrate the method used for determining the curative dose. It will be noted in table 3 that some variation occurred with different samples of the compound and in different experiments with the same sample. In general, less variation occurred when doses were administered for seven days or longer. When treatment was administered for seven doses (twice daily for three and one-half days), using Method B, the curative (CD<sub>50</sub>) intraperitoneal dose of the hydrochloride salt approximated 4 mgm. per kgm., and the curative (CD<sub>50</sub>) oral dose was about 20 mgm. per kgm. When the treatment period was extended to one or two weeks, the curative (CD<sub>50</sub>) intraperitoneal dose was 2 mgm. per kgm., and the curative (CD<sub>50</sub>) oral dose about 15 mgm. per kgm. When compared with intraperitoneal curative doses of Stilbamidine and Bayer 205 administered under similar conditions (table 7), p-phenylene diguanidine is much the least effective of the three compounds. The oral curative doses, however, present a somewhat different picture. It is five times more effective when given orally than Bayer 205, and one-sixth as effective as Stilbamidine administered orally. The ratio of intraperitoneal to oral treatment (table 7) also demonstrates the marked difference between these two routes of administration with these three compounds. For example, 500 times more Bayer 205 is required to produce cures by oral treatment than by intraperitoneal treatment, and 120 times more Stilbamidine. Only five times more p-phenylene diguanidine is required to obtain a curative oral dose than a curative intraperitoneal dose. The mode of action of any of these trypanocidal substances is thus far unknown.

In rabbits infected with *T. equiperdum*, 10 mgm. per kgm. of p-phenylene diguanidine administered intraperitoneally or intravenously, twice daily for fourteen days, will produce cures, but 5 mgm. per kgm. will not. Rabbits were inoculated intravenously with a saline suspension of trypanosomes obtained from a heavily infected mouse, and were held before treatment until symptoms appeared or until trypanosomes were found in the blood. *T. equiperdum* in rabbits

TABLE 1

Comparison of single dose and multiple dose treatment with p-phenylene diguanidine (*T. equiperdum* in mice)

DOSE I.P.  mgm./kgm.	NO. OF TREATMENTS	NO. OF ANIMALS	DEATHS—AFTER INOCULATION				ACTIVITY
			1-2 days	3-5 days	6-15 days	16-30 days	
0.2	7*	10	0	10	—	—	None
0.4	7*	10	0	10	—	—	None
0.8	7*	10	0	10	—	—	None
1.6	7*	10	0	1	9	—	Suppressive
3.0	7*	10	0	1	1	1	Curative
6.3	7*	10	0	0	0	1	Curative
Controls.....	None	10	0	10	—	—	
0.2	1†	10	0	10	—	—	None
0.4	1†	10	0	10	—	—	None
0.8	1†	10	0	8	2	—	None
1.6	1†	10	0	10	—	—	None
3.0	1†	10	0	6	4	—	Suppressive
6.3	1†	10	0	5	5	—	Suppressive
12.5	1†	10	0	1	9	—	Suppressive
25	1†	10	0	0	10	—	Suppressive
50	1†	10	0	1	7	1	Suppressive
100	1†	10	0	0	3	1	Curative
Controls.....	None	10	0	10	—	—	

\* One treatment on the first day of inoculation with trypanosomes; two treatments daily for the next three days, with approximately eight hour intervals between daily doses.

† One dose administered twenty-four hours after inoculation with trypanosomes.

is notably more resistant to treatment with arsenicals than the same parasite in mice (6).

When treatment with p-phenylene diguanidine was delayed in mice for 54 hours after inoculation with *T. equiperdum* the oral curative dose ( $CD_{50}$ ) was about 25 mgm. per kgm., and the intraperitoneal curative dose ( $CD_{50}$ ) was about 8 mgm. per kgm. (table 9). This demonstrates that when the compound is introduced shortly before the crisis or peak of parasitemia, somewhat higher doses are needed to clear the blood of trypanosomes than when treatment is initiated within a few hours after infection.

TABLE 2

*Effect of multiple treatments with p phenylene diguanidine against Trypanosoma equiperdum in mice (Series I 493)*

DOSE MG/M/KGM GRAL*	NO MICE USED	DEATHS						TOTAL DEATHS PER CENT ( WAS	EFFECT
		Weeks after inoculation							
		1	2	3	4	5	6		
								per cent	
1 T I D 15 doses	10	10						100	None
2 5 T I D 15 doses	10	10						100	None
5 T I D 15 doses	10	1	6	1	1			90	Suppressive
7 5 T I D 15 doses	10		5	3	1			90	Suppressive
10 T I D 15 doses	10		1		1			20	Curative
15 T I D 15 doses	10							0	Curative
20 T I D 15 doses	10					2		20	Curative
25 T I D 15 doses	10							0	Curative
1 T I D 33 doses	10	10						100	None
2 5 T I D 33 doses	10	10						100	None
5 T I D 33 doses	10	1	2	7				100	Suppressive
7 5 T I D 33 doses	10			7				70	Suppressive
10 T I D 33 doses	10			1	1			50	Curative
15 T I D 33 doses	10	1						10	Curative
20 T I D 33 doses	10							0	Curative
25 T I D 33 doses	10							0	Curative
1 B I D 12 doses	10	10						100	None
2 5 B I D 12 doses	10	10						100	None
5 B I D 12 doses	10	7	3					100	None
7 5 B I D 12 doses	10	2	4	1				70	Suppressive
10 B I D 12 doses	10			1				10	Curative
15 B I D 12 doses	10	1						10	Curative
20 B I D 12 doses	10	1						10	Curative
25 B I D 12 doses	10	1						10	Curative
1 B I D 25 doses	10	10						100	None
2 5 B I D 25 doses	10	10						100	None
5 B I D 25 doses	10	9	1					100	None
7 5 B I D 25 doses	10	4	3	3				100	Suppressive
10 B I D 25 doses	10	1		5				60	Suppressive
15 B I D 25 doses	10			1	3	2		60	Suppressive
20 B I D 25 doses	10		1					10	Curative
25 B I D 25 doses	10							0	Curative
Controls	37	37						100	

\* Dosage intervals explained in the text

In one experiment designed to test the efficacy of administering the compound in the diet, 50 per cent of infected mice survived for 30 days after one week's feeding on 0.125 per cent p phenylene diguanidine in the feed. Higher concen-

trations of the drug in the feed were not tolerated. The drug was mixed with finely ground food pellets and placed in small earthenware food cups available to the mice at all hours of the day and night for one week.

TABLE 3

*Estimation of curative dose of p-phenylene diguanidine in 21 different test series.  
(T. equiperdum in mice)*

SERIES NO.	COMPOUND* SAMPLE NO.	TREATMENT SCHEDULE	CURATIVE DOSE (CD <sub>50</sub> )	
			I P.	Oral
			mgm /kgm.	mgm /kgm.
E-55 ...	1	B.I.D. 3½ days	3.0	
E-102	1	B I.D. 3½ days	3.0	
E-197	2	B I.D. 3½ days	12.5	50.0
E-197	2A	B I.D. 3½ days	6.0	
E-200	2A	B I.D. 3½ days	6.0	50.0
E-200	2B	B.I.D. 3½ days	6.0	25.0
E-214	2B	B I.D. 3½ days	1.5	
E-280	2B	B.I.D. 3½ days	6.0	25.0
E-306	2B	B.I.D. 3½ days	3.0	12.5
E-417	2B	B I.D. 3½ days	4.0	20.0
E-493	2B	B.I.D. 7 days		10.0
E-495	3	B.I.D. 7 days		20.0
E-495	2B	B.I.D. 7 days		15.0
E-214	2B	B.I.D. 14 days	1.5	
E-280	2B	B I.D. 14 days	3.0	
E-493	2B	B.I.D. 14 days		20.0
E-214	2B	T I.D. 3½ days	1.5	
E-493	2B	T.I.D. 7 days		10.0
E-495	3	T I.D. 7 days		15.0
E-214	2B	T.I.D. 14 days	1.5	
E-493	2B	T.I.D. 14 days		10.0

\* Sample No. 1 = First batch, impure bicarbonate.

Sample No. 2 = Second batch, impure bicarbonate.

Sample No. 2A = Purified carbonate from No. 2.

Sample No. 2B = Purified and recrystallized hydrochloride from No. 2

Sample No. 3 = Third batch; bicarbonate 42%, carbonate 58%.

*Other guanidines and related compounds.* Eleven compounds related structurally to p-phenylene diguanidine are listed in table 4. These produced no trypanocidal effects in the doses indicated. It should be noted particularly that ortho and meta phenylene diguanidine were completely inactive. In table 5 eleven compounds are listed which showed varying degrees of effectiveness against *T. equiperdum*. Four of these (83-L, 318-L, 331-L and 340-L) possess activity which is very near to that of p-phenylene diguanidine. With

the exception of 331-L (bis-(4-guanidophenyl)-sulfide carbonate), however, these compounds are less active when administered orally than p-phenylene diguanidine. Compound 331-L is as effective as p-phenylene diguanidine, and possibly slightly more so, when administered either orally or intraperitoneally (table 6). Its activity, however, is not markedly different from the simpler p-phenylene diguanidine.

Several fuchsin-guanidine compounds were also made, because of the marked similarity in dosage and activity between basic Fuchsin and p-phenylene diguanidine. The trypanocidal activity of fuchsin dyes has been known for nearly

TABLE 4

Compounds related to p-phenylene diguanidine which show no trypanocidal activity in the doses used

COMPOUND NO	NAME	HIGHEST DOSE USED B I D—3 DAYS	
		I.P. mgm /kgm	Oral mgm /kgm
U-99	o-Phenylene diguanidine	25	200
37-L	m-Phenylene diguanidine	25	25
U-433	Phenylene-1,3,5-triguanidine	50	200
155-L	Phenylene-1,4-diurea	—	200
154-L	2-Methylphenylene-1,4-diurea	—	200
314-L	1,6-Diphenyl-p-phenylene diguanidine dihydrochloride	12.5*	200
297-L	1,6-Dilauryl-p-phenylene diguanidine dihydrochloride	—	200
372-L	p-Guanidobenzylamine dihydrochloride	50	200
320-L	1,3-Bis(4-aminophenyl)-guanidine trihydrochloride	50	200
324-L	1,3-Bis(4-ureidophenyl)-guanidine hydrochloride	50	200
338-L	Bis (4-guanidophenyl)-thiourea (impure)	6 25*	200

\* Toxic at higher doses.

fifty years. Basic Fuchsin produces cures in mice infected with *T. equiperdum* at almost exactly the same oral and intraperitoneal levels as p-phenylene diguanidine. It is considerably more toxic, however. Biguanidine and triguanidine substitutions were made on the fuchsin molecule, and the activity remained at about the same level as either of the parent structures, providing methyl groups were not added to one of the phenyl rings. Triphenylmethane-4,4',4''-triguanidine was more active than triphenylmethane-4,4'-biguanidine. Because of the toxicity of these compounds, however, investigations of the group were stopped.

DISCUSSION. The data presented with p-phenylene diguanidine demonstrate that this compound possesses at least one very real advantage over several other

known trypanocides in laboratory animals. Although it is far less effective than Bayer 205, Stilbamidine, Melarsen, and p-arseno-phenylbutyric acid when administered parenterally, it does possess curative properties at relatively low oral dosages. It differs from the arsenicals, Bayer 205, and the aromatic diamidines, in that very large single intraperitoneal doses (100 mgm. per kgm.) are required to produce cures in mice infected with *T. equiperdum*. The total curative intraperitoneal dose when administered for seven doses (4 mgm. per kgm. twice daily, a total of 28 mgm. per kgm.) is approximately one-fourth of

TABLE 5

*Compounds related to p-phenylene diguanidine which show trypanocidal activity*

COMPOUND NO.	NAME	APPROXIMATE P-PHENYLENE DIGUANIDINE EQUIVALENT	
		I.P.	Oral
350-L.....	p-Phenylenedibiguanide monohydrate	0.08	<0.05
253-L.....	2-Chloro-1,4-phenylene diguanidine dihydrochloride	0.16	0.05
369-L.....	2-Chloro-1,4-phenylene dibiguanide dihydrochloride	0.16	<0.1
291-L.....	2-Methyl-1,4-phenylene diguanidine dihydrochloride	0.08	0.05
251-L.....	1,6-Diisopropyl-p-phenylenediguanidine dihydrochloride	0.08	<0.05
83-L.....	Diphenylurea-4,4'-diguanidine carbonate	1.0 plus	0.2
318-L.....	1,3-Bis(4-guanidophenyl)-guanidine trihydrochloride	1.0 plus	0.3
331-L.....	Bis-(4-guanidophenyl)- sulfide carbonate	1.0 plus	1.0
353-L.....	Bis-(4-guanidophenyl)-sulfoxide dihydrochloride	0.16	<0.1
325-L.....	Bis-(4-guanidophenyl)-sulfone dihydrochloride	<0.08	<0.05
340-L.....	3,3'-Dimethyl-4,4'-diguanido bi-phenyl	1.0 plus	0.3

the single dose needed to produce cures. The chemotherapeutic indices given for p-phenylene diguanidine when administered in multiple doses (table 6) are lower than those which have been reported for Melarsen (5), p-arseno-phenylbutyric acid (6) and Stilbamidine (3) when administered in a single dose.

The fact that few guanidines related to p-phenylene diguanidine showed significant trypanocidal activity was rather surprising. Although all possible deviations in structure were not investigated, a sufficient number of related derivatives were tested to demonstrate that p-phenylene diguanidine *per se* probably possesses the maximum potentialities in this particular group of guanidines.

Chronic toxicity studies will be helpful for evaluating the potentialities of p-phenylene diguanidine for clinical use in man. Some studies have been under-

TABLE 6

Comparison of four guanidines by the multiple dose technique (Series E 417)  
(T equiperdum in mice)

COMPOUND	DOSAGE	APPROXIMATE CURATIVE DOSE (CD <sub>10</sub> ) MG/M/KGM		APPROXIMATE LETHAL DOSE (LD <sub>10</sub> ) MG/M/KGM		CHEMOTHERAPEUTIC INDEX LD <sub>10</sub> /CD <sub>10</sub>	
		I P	Oral	I P	Oral	I P	Oral
p Phenylene diguanidine	B I D 3½ days	4 0	20 0	40 0	175 0	10	8 7
1,3 Bis(4 guanidophenyl) guanidine tri HCL	B I D 3½ days	2 0	60 0	20 0	400 0	10	6 6
Bis (4 guanidophenyl) sulfide CO <sub>2</sub>	B I D 3½ days	2 0	20 0	50 0	175 0	25	8 7
3,3'-Dimethyl-4,4'-diguanide biphenyl	B I D 3½ days	2 0	60 0	20 0	80	10	1 3

TABLE 7

Comparison of Stilbamidine, Bayer 205 and four guanidine derivatives Multiple dose technique (T equiperdum in mice)

COMPOUND	APPROXIMATE SUPPRESSIVE DOSE (M Th D) MG/M/KGM B.I.D FOR 3½ DAYS		APPROXIMATE CURATIVE DOSE (CD <sub>10</sub> ) MG/M/KGM B.I.D FOR 3½ DAYS		RATIO OF I P TO ORAL TREATMENT	
	I P	Oral	I P	Oral	M Th.D	CD <sub>10</sub>
Stilbamidine	0 0175	1 6	0 025	3 0	1 91	1 120
Bayer 205	0 15	50 0	0 2	100 0	1 333	1·500
p Phenylene diguanidine	2 0	10 0	4 0	20 0	1 5	1 5
1,3 Bis(4 guanidophenyl) guanidine Tri HCL	1 0	45 0	2 0	60 0	1 45	1 30
Bis (4 guanidophenyl) sulfide CO <sub>2</sub>	1 0	10 0	2 0	20 0	1 10	1 10
3,3'-Dimethyl-4,4'-diguanide biphenyl	1 0	20 0	2 0	60 0	1 20	1 30

TABLE 8

Comparison of three arsenicals by the single dose technique  
(T equiperdum in mice)

COMPOUND	PARENTERAL CURATIVE DOSE (CD <sub>10</sub> )	PARENTERAL LETHAL DOSE (LD <sub>10</sub> )	CHEMOTHERAPEUTIC INDEX LD <sub>10</sub> /CD <sub>10</sub>
	mgm/kgm	mgm/kgm	
Tryparsamide	940*	3,750*	4
	1000†	4,000†	4
Melarsen	0 5†	17 5†	35
p Arsenoso phenylbutyric acid	1 6*	33*	21

\* Eagle *et al*, 1944

† Weinman, 1946



taken with regard to the distribution of the compound in the central nervous system of laboratory animals, but the occurrence of other guanidines in normal blood and body fluids interfered with quantitative determinations.

No effect has been demonstrated by p-phenylene diguanidine or related derivatives against *Leishmania donovani* in hamsters, *Schistosoma mansoni* in mice, *Litomosoides carinii* in cotton rats, *Plasmodium lophurae* in ducks or *Trypanosoma cruzi* in mice.

TABLE 9

Effect of p-phenylene diguanidine against *T. equiperdum* when dosage is started fifty-four hours after inoculation

DOSE	NO. OF TREATMENTS*	NO. OF MICE	DEATHS—DAYS AFTER INOCULATION				ACTIVITY
			1-2 days	3-5 days	6-15 days	16-30 days	
mgm./kgm.							
5.0 oral	13	10		10			None
10.0 oral	13	10		10			None
15.0 oral	13	10		7	1	2	Suppressive
20.0 oral	13	10		6		3	Suppressive
25.0 oral	13	10		1		3	Curative
1.0 Ip.	13	10		10			None
2.0 Ip.	13	10		9		1	None
4.0 Ip.	13	10		2	1	3	Suppressive
8.0 Ip.	13	10		1		1	Curative
None	Controls	10		10			

\* Treated twice daily for seven days with the exception of the sixth day when a single dose was administered.

## SUMMARY

Data are given which demonstrate the trypanocidal activity of p-phenylene diguanidine and related derivatives against *Trypanosoma equiperdum* in mice. This compound is much less effective when administered parenterally than arsenicals and other known trypanocides such as Bayer 205 or Stilbamidine. It is effective when administered in multiple doses orally, however, and for this reason is considered to be of potential interest for trial in man or domestic animals. Of the related guanidines tested none showed markedly greater activity or were better tolerated than p-phenylene diguanidine.

## REFERENCES

1. SAFIR, S., KUSHNER, S., SMITH, V., BRANCONE, L., AND SUBBAROW, Y.: J. Org. Chem., 13: 924, 1948.
2. KING, H., LOURIE, E., AND YORKE, W.: Lancet, 233: 1360, 1937.
3. LOURIE, E., AND YORKE, W.: Ann. Trop. Med., 33: 289, 1939.
4. ST. A. HEATHCOTE, R.: J. Trop. Med. and Hyg., 49: 1, 33, 1946.
5. WEINMAN, D.: Am. J. Trop. Med., 26 (Supp.): 95, 1946.
6. EAGLE, H., HOGAN, R., DOAK, G., AND STEINMAN, H.: Public Health Reports, 59: 765, 1944.

# THE ANALEPTIC EFFECT OF SODIUM SUCCINATE IN BARBITURATE ANESTHESIA IN RABBITS

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In 1943 Soskin and Tabenhaus first reported upon the use of sodium succinate in controlling barbiturate anesthesia (1). These authors presented their work as an extension of the observations of Quastel and Wheatley (2) upon the ability of succinate to support the metabolism of brain tissue poisoned by barbiturates *in vitro*. They concluded that sodium succinate exerted a protective effect against toxic doses of pentobarbital in rats and further that it would shorten the duration of both sodium amytal and sodium pentobarbital anesthesia.

Since this time, several reports have appeared with conflicting findings as to the effectiveness of succinate in controlling the duration of barbiturate anesthesia and in reducing the toxicity of barbiturates. In addition there has been some disagreement as to the possible mode of action of the succinate salt.

Beyer and Latven working with mice and rats (3) could not obtain striking confirmation of the effect of sodium succinate upon shortening the duration of pentobarbital hypnosis. However, upon using much higher doses of the analeptic they were able to demonstrate a moderate effect.

Pinschmidt, Ramsey and Haag (4) using rats, mice and rabbits obtained somewhat less striking results than those of Soskin and Tabenhaus. However, they demonstrated that premedication of rats both shortened the duration of pentobarbital anesthesia and afforded protection against lethal doses.

On the basis of experience with humans receiving sodium Pentothal as an operative anesthetic, Barrett (7) concluded that succinate as the disodium hexahydrate was an efficient analeptic in barbiturate depression.

Corson, Koppanyi and Vivino (6), working with dogs, rabbits, cats and rats, found that neither disodium succinate nor disodium fumarate exerted any life saving effects when the animals were poisoned either with long or short acting barbiturates. They further demonstrated that whether in small or large doses these drugs did not alter the duration of anesthesia.

Lardy, Hansen and Phillips (5) were not able to confirm the effect of either intramuscular or intraperitoneal sodium succinate in shortening the duration of either sodium amytal or sodium pentobarbital anesthesia in rats.

The rationale which has been advanced by the proponents of sodium succinate has been its ability to support respiration *in vitro* of tissue poisoned with barbiturate, and hence to act as a source of metabolic energy for the breakdown of the barbiturate. We have attempted to evaluate the action of sodium succinate upon the metabolism of the barbiturate *in vivo*.

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The development of satisfactory analytical methods for barbiturates using small samples of blood has made feasible the evaluation of the overall rates of metabolism of these compounds.

**METHODS.** Female albino rabbits of about 2 kgm. were used throughout this study. Food but not water was withheld for from 16 to 18 hours before each experiment. Blood specimens of 5 cc. were obtained by cardiac tap. All animals received the barbiturates by injection in the marginal ear vein.

All solutions were prepared daily. The sodium succinate, sodium pentobarbital and thiopental sodium (Pentothal) were each prepared from crystalline material and diluted with sterile distilled water.

Analyses were performed in duplicate. The values presented are averages of the two determinations. The method of Goldbaum (8) was used for the determination of sodium pentobarbital. The method of Jailer and Goldbaum was used for the determination of sodium Pentothal (9).

The awakening time was taken to be the instant that the animal spontaneously assumed the prone position and gathered its legs beneath its body. Reaction time was determined as the instant the animal would assume the prone position in response to a standard pinch stimulus applied to the tip of the ear.

**EXPERIMENTAL.** The first experiment was designed as a single cross-over to evaluate the effect of intravenous sodium succinate in a dosage of 500 mgm./kgm. upon the duration of sodium pentobarbital anesthesia. Sodium pentobarbital, 35 mgm./kgm., was injected at a rate of 1 cc. (20 mgm./cc.) per minute in the marginal ear vein. Ten minutes after the completion of the injection, a blood sample was withdrawn for analysis. Immediately sodium succinate, 500 mgm./kgm., was injected in the marginal ear vein at the rate of 0.5 cc./minute (250 mgm./cc.). Blood samples were then withdrawn at 30 minutes, upon reacting and upon awakening. Each animal was given a ten-day rest period before the cross-over was performed. The animals were run in groups of four, two subject and two control. The data are presented in table I.

There appears to be slight difference between the animals as subjects or as controls with respect to reaction time, sleeping time, or blood level at the time of awakening. On analysis by Student's "*t*" test, the value computed for the reaction time is  $t = 0.275$ ,  $p = 0.41$ , while that for the total sleeping time is  $t = 0.236$ ,  $p = 0.40$ . Thus there is no significant difference in the behavior of this group of animals under pentobarbital anesthesia with or without subsequent intravenous injection of a moderate dose of sodium succinate. Furthermore, it is evident that the awakening blood level fluctuates through a narrow range with a mean of 1.2 mgm. per cent. We have found the blood level of pentobarbital sodium on awakening to be quite constant at  $1.16 \pm 0.04$  mgm. per cent in the rabbit (10). Therefore, the administration of sodium succinate in this dosage does not enable the animal to awake with a greater quantity of pentobarbital in the circulating blood than in the absence of the analeptic. In figure 1 we have plotted the blood levels (on the  $\log_{10}$  scale) against the time in minutes. There is no significant variation in the scatter of points between the subject and control series. Moreover, regression lines based upon mean values have the same slope.

Hence, the rate of metabolism of pentobarbital as judged by its rate of disappearance from the blood stream is not influenced by sodium succinate in this dosage, given intravenously ten minutes after the administration of the barbiturate.

TABLE 1

*Effect of intravenous sodium succinate 500 mgm./kgm. upon sodium pentobarbital anesthesia (Single cross over at ten-day interval)*

RABBIT	SUBJECT			RABBIT	CONTROL		
	Reaction time	Sleeping time	Awakening level		Reaction time	Sleeping time	Awakening level
	minutes	minutes	mgm. per cent		minutes	minutes	mgm. per cent
P166	165	210	1.5	P166.....	63	73	1.1
D1	50	60	1.0	D1.....	64	118	1.3
M70.	75	115	1.3	M70.....	70	90	1.1
M3 ...	85	105	1.4	M3.....	61	89	1.1
O20	63	93	1.1	O20.....	40	—	1.2
#95	62	91	1.1	#95.....	64	84	1.3
M38	48	89	1.1	M38.....	63	93	1.2
#4	90	150	0.8	#4.....	125	180	0.8
Mean ..	78.5	114.5	1.2	Mean.....	68.7	103.8	1.1

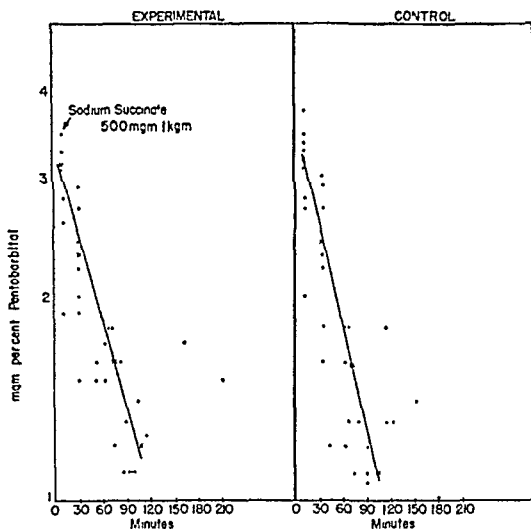


FIG. 1. EFFECT OF INTRAVENOUS SODIUM SUCCINATE UPON THE RATE OF DISAPPEARANCE OF PENTOBARBITAL FROM THE CIRCULATING BLOOD

In order to obviate the possibility of having influenced the results by the repeated cardiac taps the following experiment was performed. Each of twenty rabbits received 35 mgm./kgm. of an aqueous solution of sodium pentobarbital

in the marginal ear vein. Ten minutes following injection the subject group received 500 mgm./kgm. sodium succinate intravenously. Reaction time and sleeping time were noted as in the previous experiment. A blood sample was withdrawn upon the awakening of each animal. The results of this experiment are summarized in table 2.

Again, there was no difference demonstrated in reaction time, sleeping time, or awakening blood level between the subject and the control group. On subjecting the data to the analysis of variance an F ratio below 0.20 was found in each category. This is insignificant at the 1 per cent level.

*Effect of Higher Dosage of Succinate:* Inasmuch as higher dosages had seemed to produce significant shortening of anesthesia in the hands of other workers,

TABLE 2

*Effect of intravenous sodium succinate 500 mgm./kgm. upon anesthesia following a single injection of intravenous sodium pentobarbital 35 mgm./kgm.*

SUBJECT			CONTROL		
Reaction time	Sleeping time	Awakening blood level	Reaction time	Sleeping time	Awakening blood level
minutes	minutes	mgm. per cent	minutes	minutes	mgm. per cent
40	60	1.1	110	175	1.1
37	55	1.4	62	84	1.2
62	85	1.2	48	72	1.0
58	80	0.9	67	95	1.4
104	135	1.1	45	68	0.8
60	95	1.3	50	76	1.6
75	102	1.0	75	104	1.1
90	110	1.1	84	98	1.1
98	210	1.6	80	102	1.3
62	90	1.0	88	112	1.2
Mean: 68.6	101.2	1.2	70.9	98.6	1.2

we next determined the maximal dosage of sodium succinate which was tolerated by rabbits intravenously without any signs of toxicity. A dose of 2000 mgm./kgm. was well tolerated if the injection was made over the course of eight to ten minutes. Above this level rabbits exhibited signs of muscular twitching, culminating in generalized convulsions when given 2500 mgm./kgm.

The first two experiments were then repeated using a dose of 2000 mgm./kgm. of sodium succinate in the subject group. Due to the large volume of fluid required to deliver this dose (8 to 10 cc.) the control groups were given isotonic sodium chloride as a fluid volume control. The data are presented in tables 3 and 4.

In the cross-over experiment no demonstrable difference in either reaction time or sleeping time was found between the group as control or as subject. On analysis by Student's "t" test the value for reaction time is  $t = 0.99$ ,  $p = 0.17$ ; and for the sleeping time,  $t = 0.45$  and  $p = 0.32$ . There was no significant

difference between the awakening blood levels of the two groups. It is of interest that the two animals which fall at the extremes of the range (No 4 and M96) do so when subject and when serving as their own controls. As in the first experiment, when the data are plotted as  $\log_{10}$  blood level against time, straight lines of the same slope are obtained for both the subject and control group.

TABLE 3

*Effect of intravenous sodium succinate 2000 mgm /kgm upon sodium pentobarbital anesthesia (Single cross over at ten day interval)*

RABBIT	SUBJECT			RABBIT	CONTROL		
	Reaction time	Sleeping time	Awakening level		Reaction time	Sleeping time	Awakening level
	minutes	minutes	mgm per cent		minutes	minutes	mgm per cent
292	65	95	1.3	292	80	120	1.1
M90	136	186	1.4	M90	120	145	1.0
No 4	58	92	1.9	No 4	60	100	1.5
M96	118	173	0.9	M96	75	135	0.8
000	75	100	1.1	000	68	125	1.0
Mean	90.4	129.2	1.3	Mean	80.6	125	1.1

TABLE 4

*Effect of intravenous sodium succinate 2000 mgm /kgm upon anesthesia following a single injection of intravenous sodium pentobarbital 35 mgm /kgm*

SUBJECT		CONTROL	
Sleeping time	Awakening level	Sleeping time	Awakening level
minutes	mgm per cent	minutes	mgm per cent
130	1.3	145	1.1
125	1.2	165	1.1
125	1.2	205	1.0
140	1.0	145	1.2
140	0.7	110	1.1
175	1.0	225	1.0
80	1.5	125	1.4
120	1.5	105	1.4
165	0.9	120	1.1
145	1.1	125	1.1
130	1.0	145	1.0
Mean 134	1.14	146.8	1.14

From table 4 no difference can be established between the subject and control group. In this experimental group all sources of stimulation were rigidly excluded from the time of injection of the analeptic or of the saline control, until the spontaneous awakening. These data, when subjected to the analysis of variance, yield an F ratio of 0.72.

Therefore, it appears that the intravenous administration of 2000 mgm./kgm. of sodium succinate ten minutes after the onset of pentobarbital anesthesia is without effect either upon the duration of anesthesia or upon the rate of metabolism of the pentobarbital as determined by its disappearance from the circulating blood.

*Intramuscular Administration of Succinate.* Since there remains the possibility that due to rapid elimination, the intravenous injection of a large quantity

TABLE 5  
*Effect upon sodium pentobarbital anesthesia of intramuscular sodium succinate 1000 mgm./kgm.*

SUBJECT		SODIUM FUMARATE CONTROL		HYPERTONIC SALINE CONTROL	
Sleeping time	Awakening level	Sleeping time	Awakening level	Sleeping time	Awakening level
<i>minutes</i>	<i>mgm. per cent</i>	<i>minutes</i>	<i>mgm. per cent</i>	<i>minutes</i>	<i>mgm. per cent</i>
97	1.3	120	1.0	180	1.2
Expired	—	Expired	—	Expired	—
180	0.9	152	1.5	175	1.1
120	1.1	193	1.1	143	1.0
200	0.9	195	0.9	140	1.1
Mean: 149	1.1	165	1.1	159	1.1

2nd replication

SUBJECT		SODIUM FUMARATE CONTROL		HYPERTONIC SALINE CONTROL	
Sleeping time	Awakening level	Sleeping time	Awakening level	Sleeping time	Awakening level
<i>minutes</i>	<i>mgm. per cent</i>	<i>minutes</i>	<i>mgm. per cent</i>	<i>minutes</i>	<i>mgm. per cent</i>
Expired	—	Expired	—	Expired	—
86	1.2	68	1.1	130	1.2
123	1.7	230	1.1	138	1.3
120	1.4	160	1.3	Expired	—
125	1.2	125	1.5	205	1.1
Mean: 114	1.4	145	1.2	157	1.2

of sodium succinate might lead only to an evanescent period of high concentration in the blood, the effect of intramuscular injection of the analeptic was investigated.

Fifteen animals were distributed among three groups. Each group received 35 mgm./kgm. of sodium pentobarbital intravenously. Ten minutes after the onset of anesthesia the animals in the subject group received 1000 mgm./kgm. of sodium succinate in the thigh muscles. There were two control groups, the first receiving 1000 mgm./kgm. sodium fumarate, and the second receiving 700 mgm./kgm. sodium chloride. The concentration of the solutions was such that the animals received equal volumes of fluid. The experiment was repeated on the succeeding day upon a second group of animals. The data are presented in table 5.

There were no significant differences noted either between the subject and the control groups or between the two replications. An F ratio of less than 2 was obtained in each instance.

*Pentothal.* In attempting to evaluate the effect of succinate upon sodium Pentothal anesthesia, 26 rabbits were distributed among six groups. Each

TABLE 6

*Effect of intravenous injection of sodium succinate, sodium chloride and water upon anesthesia following a single intravenous injection of Pentothal 15 mgm./kgm.*

NUMBER OF RABBITs	ANTIDOTE	TIME SLEPT	AWAKENING LEVEL
		minutes	mgm. per cent
3	Sodium succinate, 1000 mgm./kgm., I.V., 2 min. following Pentothal	25	1.45
		19	1.70
		30	1.50
5	Sodium succinate, 2000 mgm./kgm., IV., 5 min. following Pentothal	31	2.00
		38	1.45
		19	1.25
		29	1.45
		26	0.95
5	Sodium succinate, 1000 mgm./kgm., I.V., together with Pentothal	30	1.60
		62	1.35
		56	1.05
		40	1.45
		Died	—
3	None	37	1.40
		27	1.25
		19	1.40
5	Water, 4 cc./kgm., I.V., 5 min. following Pentothal	31	1.55
		47	0.45
		24	1.25
		38	0.80
		12	—
5	Sodium chloride, 400 mgm./kgm., I.V., together with the Pentothal	62	1.00
		77	1.05
		36	1.00
		43	1.60
		27	1.35

animal received 25 mgm./kgm. of Pentothal intravenously in the form of a freshly prepared 5 per cent solution. The injections were completed within two minutes. A second intravenous injection of sodium succinate, saline or water, was given as indicated in table 6.

Varying the time of injection or the doses of the analeptic did not significantly alter the duration of the anesthesia as compared with the controls. Upon subjecting the data to the analysis of variance, an F ratio of 2.24 is obtained



for duration of anesthesia; an F ratio of 1.33 is obtained for the awakening blood levels. Therefore, at the 1 per cent level there are no significant differences in the effects of the various treatments upon the duration of anesthesia produced by a single injection of 25 mgm./kgm. of sodium Pentothal.

A study of brain tissue Pentothal levels was accomplished. The dose of Pentothal was 25 mgm./kgm. administered as before. Sodium succinate, 1000 mgm./kgm. was given intravenously two minutes after the completion of the Pentothal injection. The controls received no additional medication. Blood levels were determined at ten-minute intervals until awakening. Upon awakening the animals were killed with an intravenous injection of 10 cc. of air and the brains were removed for analysis. The data from one such experiment using three subject and three control animals are presented in figure 2. The brain level of the barbiturate was expressed in terms of mgm./100 gm. tissue. The regression lines for both subject and control groups have approximately the same slope. The level of the drug found in the brain in both groups does not significantly differ. Therefore, in this series the rate of metabolism of the drug does not appear to be affected by the analeptic, nor does the animal recover in the presence of an increased amount of barbiturate in the brain.

**DISCUSSION.** The data presented demonstrate that the administration of both large and small doses of sodium succinate does not affect the metabolism of sodium pentobarbital and sodium Pentothal in rabbits as determined by the rate of disappearance of the barbiturate from the circulating blood.

The positive effects, found by previous workers, of shortening of the duration of anesthesia had been supposed to be due to the influence of the succinate upon the intermediary metabolism of the barbiturate. In the light of some recent data this supposition may be without adequate foundation.

Lardy *et al.* (5) in their communication have pointed out that, while the tissue when poisoned with barbiturate was undoubtedly able to metabolize succinate as judged by oxygen consumption, no evidence had been presented to show that this oxidation was coupled with phosphorylation. In view of their previous inability to demonstrate the coupling with phosphorylation in *in vitro* preparations, which rapidly oxidized succinate, doubt was expressed as to the ability of the succinate to act as a source of metabolic energy. However, the recent communication of Eiler and McEwen (11) indicates that under certain conditions in brain homogenates succinate oxidation may coincide with phosphorus uptake.

Furchgott and Shorr (12), investigating the respiration of various tissues, including brain, under conditions of low oxygen tension, found that succinate in substrate concentrations was able to increase markedly the rates of oxygen consumption. However, the energy from the increased oxygen consumption was found to be incapable of increasing the rates of metabolic processes, such as acetylcholine synthesis in brain or urea synthesis in liver, which are depressed under conditions of low oxygen tension. Moreover, Klein and Olsen (13), studying the distribution of some of the 3- and 4-carbon respiratory inter-

mediates, found that the rate of transfer of succinate between the arterial plasma and the cerebral hemispheres of the cat was always less than that of glucose. They therefore postulated that succinate would not replace glucose in maintaining central nervous system function.

Hence, the probability of succinate serving as a source of energy by means of which the barbiturate would be metabolized seems unlikely. The failure of the succinate to effect a more rapid removal of barbiturate from the circulating blood would appear to support this contention.

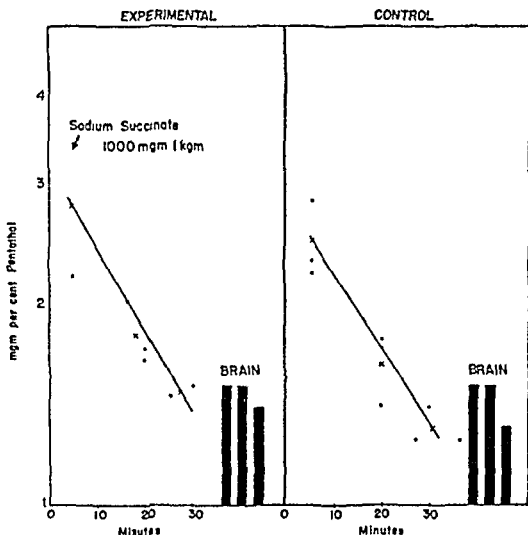


FIG. 2. EFFECT OF INTRAVENOUS SODIUM SUCCINATE UPON THE DISAPPEARANCE OF PENTOTHAL FROM THE BRAIN AND BLOOD OF THE RABBIT

Koppanyi (6, 14) has demonstrated that a moderate diuresis does not significantly affect the duration of anesthesia of barbital whether achieved by means of glucose solutions, salts, such as ammonium chloride, or by sodium succinate. Leifer *et al.* (15) working with  $C^{14}$  have demonstrated in mice that pentobarbital does not appear in the urine. Hence, if pentobarbital and not a degradation product is the specific anesthetic agent, any diuresis achieved with sodium succinate would be unlikely to affect the duration of anesthesia.

The possibility remains that sodium succinate may act as a respiratory stimulant. Heymans (16) and co-workers have shown that sodium succinate causes

an increase in pulmonary ventilation which is maintained after section of the vagus and carotid sinus nerves or destruction of the cardio-aortic region and the cardiac sinus. Bovet *et al.* (17) obtained similar results in their preparations. However, the sodium salts of aspartic, malic, fumaric, and carbonic acids were also found to be effective. This increase in respiratory rate and depth was observed in our material. The significance of this observation is not apparent inasmuch as, despite the increased pulmonary ventilation, the duration of anesthesia and the metabolism of the drug were unaffected.

It has been urged that sodium succinate be used as an analeptic on the basis of its lack of toxicity. Recently there has been reported depression of cardiac action by succinate in the rabbit heart muscle preparation (18). Furthermore, this is a disodium salt and roughly 250 mgm. of sodium are furnished in each gram of sodium succinate. Consequently at high dosages of the drug, such as 200 cc. of an 11 per cent solution, large amounts of sodium are being given. Hence, the possible deleterious effects of this substance, particularly in such salt sensitive individuals as cardiacs, should not be disregarded.

#### CONCLUSIONS

1. The effect of sodium succinate upon duration of anesthesia due to either sodium pentobarbital or sodium Pentothal has been investigated in rabbits.
2. The duration of anesthesia has been found not to be affected.
3. The blood level at which spontaneous awakening will occur has been found to be unaffected.
4. The rate of disappearance of the barbiturate from the blood stream has been found to be uninfluenced by the administration of sodium succinate.

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#### REFERENCES

1. SOSKIN, S., AND TABENHAUS, M.: *THIS JOURNAL*, **78**: 49, 1943.
2. QUASTEL, J. H., AND WHEATLEY, A. H. M.: *Proc. Roy. Soc. London*, **B112**: 60, 1932.
3. BEYER, K. H., AND LATVEN, A. R.: *THIS JOURNAL*, **81**: 203, 1944.
4. PINSCHMIDT, N. W., RAMSEY, H., AND HAAG, H. B.: *THIS JOURNAL*, **83**: 45, 1945.
5. LARDY, H. A., HANSEN, R. G., AND PHILLIPS, P. H.: *Proc. Soc. Exper. Biol. & Med.*, **55**: 277, 1944.
6. CORSON, S. A., KOPPANYI, T., AND VIVINO, E. A.: *Anesth. & Analg.*, **24**: 177, 1945.
7. BARRETT, R. H.: *Anesth. & Analg.*, **26**: 74, 105, 1947.
8. GOLDBAUM, L.: *THIS JOURNAL*, **94**: 68, 1948.
9. JAILER, J., AND GOLDBAUM, L.: *J. Lab. & Clin. Med.*, **31**: 1344, 1946.
10. GOLDBAUM, L., AND SCHACK, J. A.: Unpublished results.
11. EILER, S. J., AND McEWEN, W. K.: *Arch. Biochem.*, **20**: 163, 1949.
12. FURCHGOTT, R. F., AND SHORR, E.: *J. Biol. Chem.*, **175**: 201, 1948.
13. KLEIN, J. R., AND OLSEN, N. S.: *J. Biol. Chem.*, **167**: 1, 1947.
14. KOPPANYI, T., MURPHY, W. S., AND KROP, S.: *THIS JOURNAL*, **52**: 223, 1934.
15. LEIFER, E.: Personal communication.
16. HEYMANS, C., AND JACOB, J.: *Experientia*, **3**: 294, 1947.
17. BOVET, D., MONOD, J., AND LWOFF, A.: *Compt. Rend. Acad. d. Sci.*, **224**: 1844, 1947.
18. FORSSMAN, S., AND LINDSTEN, T.: *Acta Pharmacol. Toxicol.*, **2**: 329, 1940.

# THE EFFECT OF LOCAL ANESTHETICS ON THE RESPIRATION OF BRAIN HOMOGENATES<sup>1</sup>

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Quastel and his associates (1, 2, 3) and more recent investigators (4, 5) have found that many central nervous system depressants including ether, urethane, barbiturates, morphine and synthetic analgesics depress the *in vitro* respiration of brain tissue. These investigations have resulted in the theory that narcosis is primarily due to the inhibition of enzyme systems necessary for the oxidation of carbohydrates in the brain. In contrast to the extensive research on the effect of central nervous system depressants on enzyme systems very limited work has been done on the effect of local anesthetics on the respiration of nerve tissue. Niwa (6) found cocaine depressed carbon dioxide production in the sciatic nerve of the frog. In 1932 Sherif (7) reported that procaine and cocaine inhibited respiration in the sciatic nerve of the rabbit. In view of the widespread use of local anesthetics for surface, regional, and spinal anesthesia, an investigation of their *in vitro* action on the respiration of nerve tissue was considered desirable at this time.

Recent developments in the use of fortified homogenates make it possible to investigate the effect of drugs on highly active and specific enzyme systems. The homogenate methods are based on the fact that the tissue is diluted until endogenous activity is reduced to a very low level. Then by the addition of water soluble components such as hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide (DPN), cytochrome c, nicotinamide and certain inorganic ions, activity in the presence of a selected substrate is restored to a high level. Brain homogenates were used in this investigation instead of homogenates from peripheral nerves because this tissue is more readily available in the quantities required for multiple Warburg experiments. The following substrates and reactions have been used to study the effect of local anesthetics on the respiration of brain homogenates and to isolate partially the site of action: (a) oxygen uptake by the homogenate in the presence of glucose, which requires both the glycolytic and oxidative enzyme chains for this reaction; (b) the anaerobic glycolysis of glucose which requires only the glycolytic enzymes; (c) the oxidation of succinate by molecular oxygen which requires the dehydrogenase, an unknown factor linking cytochrome c and b (8), the cytochromes and cytochrome oxidase; (d) the oxidation of ascorbate which requires only cytochrome c and cytochrome oxidase; (e) the anaerobic reduction of methylene blue by succinate which is catalyzed by succinic dehydrogenase; (f) the oxida-

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tion of reduced cytochrome c by molecular oxygen which is catalyzed by cytochrome oxidase; (g) the reduction of oxidized cytochrome c by succinate which requires the unknown factor and succinic dehydrogenase.

**METHODS.** Local anesthetics used in this investigation were cocaine, USP; procaine, USP; metycaine, NNR; tetracaine, USP; and nupercaine, NNR as the hydrochlorides and butacaine sulfate, USP. All molar concentrations are expressed in terms of the free alkali-

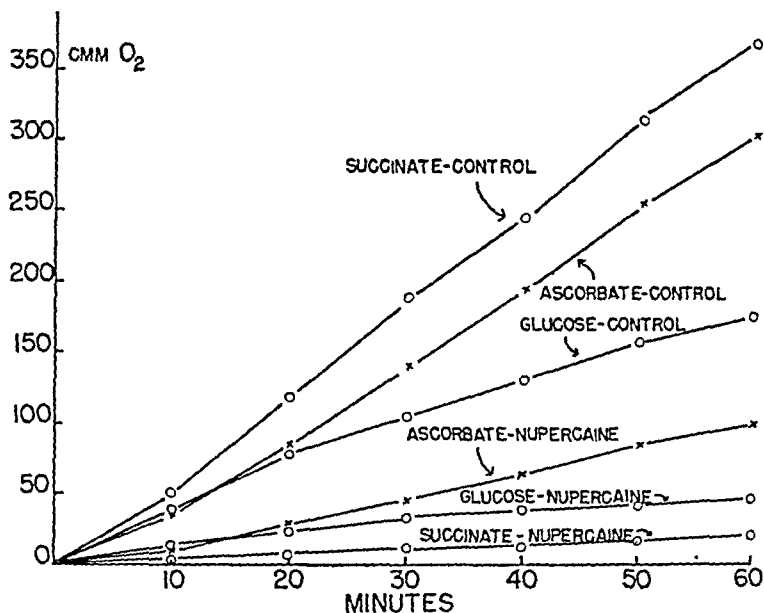


FIG. 1. EFFECT OF NUPERCaine ON THE RESPIRATION OF BRAIN HOMOGENATES

Temperature, 36.3° C.; gas phase, air; nupercaine, 0.005 *M*; concentration of solutions: (a) Glucose oxidation—glucose, 0.028 *M*; hexose diphosphate·Mg, 0.005 *M*; potassium fumarate, 0.0016 *M*; adenosine triphosphate, 0.0007 *M*; DPN, 0.001 *M*; nicotinamide, 0.01 *M*; cytochrome c, 0.00006 *M*; K<sub>2</sub>HPO<sub>4</sub>—KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4, 0.04 *M*; 0.3 cc. 1:5 rat brain homogenate in side arm; final volume, 1.0 cc. (b) Succinate oxidation—sodium succinate, 0.05 *M* from side arm; cytochrome c, 0.00017 *M*; CaCl<sub>2</sub>, 0.0004 *M*; AlCl<sub>3</sub>, 0.0004 *M*; Na<sub>2</sub>HPO<sub>4</sub>—HCl buffer, pH 7.4, 0.03 *M*; 0.3 cc. 1:4 rat brain homogenate; final volume, 3.0 cc. (c) Ascorbate oxidation—sodium ascorbate, 0.0114 *M* from side arm; cytochrome c, 0.00017 *M*; CaCl<sub>2</sub>, 0.0004 *M*; AlCl<sub>3</sub>, 0.0004 *M*; Na<sub>2</sub>HPO<sub>4</sub>—HCl buffer, pH 7.4, 0.03 *M*; 0.3 cc. 1:4 rat brain homogenate; final volume, 3.0 cc.

loid. The rate of oxygen uptake or carbon dioxide evolution was followed in Warburg manometers at 36.3 C. After a ten-minute period for gassing and temperature equilibration the stopcocks were closed, homogenate and substrate mixed and readings made at ten-minute intervals for 60 minutes. An apparatus with fourteen manometers was used in order that duplicate controls and five concentrations of any one drug could be examined simultaneously. In experiments to compare the relative effect of the anesthetics duplicate determinations were made on control vessels and on vessels with five of the anesthetics at a concentration of 0.005 *M*. This eliminated any possibility of variations due to differences in the homogenate preparation.

With minor modifications the method of Utter, Wood and Reiner (9) was used for anaerobic glycolysis, that of Reiner (10) for the oxidation of glucose and that of Schneider and Potter (11) for oxidation of succinate and ascorbate. The amount of homogenate was varied until a highly active preparation was obtained. The concentration of solutions

used, the additions to the homogenate and the activity of the preparation with time are shown in figs 1 and 3

In a few of the anaerobic glycolysis experiments lactic acid determinations were made on the contents of the Warburg vessels immediately after the 60 minute experiment by the method of Edwards (12). The effect of nupercaine on the oxidation and reduction of cytochrome c was determined by following the rate of appearance or disappearance of reduced cytochrome c with a Beckman spectrophotometer at 550 m $\mu$  (13). Succinic dehydrogenase activity was determined by the rate of reduction of methylene blue using the Thunberg method. Ninety per cent methylene blue reduction, determined visually, was used as the end point.

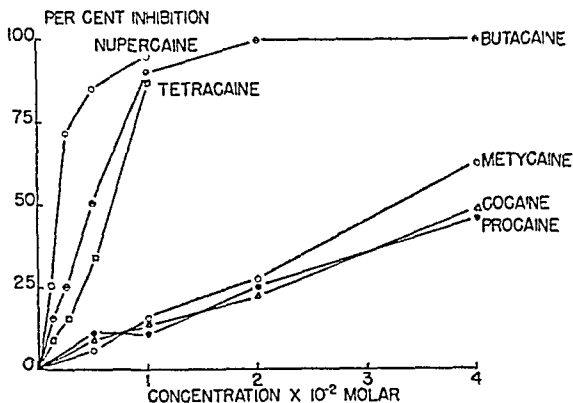


FIG 2 EFFECT OF VARYING THE CONCENTRATION OF LOCAL ANESTHETICS ON SUCCINATE OXIDATION

Experimental conditions and solution concentrations same as those given for succinate oxidation in fig 1

Tissue homogenates were prepared by grinding fresh whole rat brain in cold buffer or distilled water (14) for all experiments except those for some of the succinate and ascorbate oxidation determinations. Frozen beef brain stored on dry ice (15) was used for these experiments. Control determinations showed homogenates prepared from the beef brain and rat brain gave comparable results when succinate and ascorbate were used as substrates.

**RESULTS** The effect of nupercaine on the oxidation of glucose, succinate and ascorbate is shown in fig 1. These are typical experiments and show that oxygen uptake by the fortified homogenates is linear with time for the 60 minute period. Nupercaine produced the greatest inhibition of any of the local anesthetics. This inhibition is typical of all the drugs in that it is uniform throughout the experiment. The comparative effect of six local anesthetics at a concentration of 0.005 M on the oxidation of glucose, succinate and ascorbate is given in table 1. Depression of succinate oxidation over a range of concentrations is shown in fig 2. The effect of procaine, cocaine, and nupercaine on the

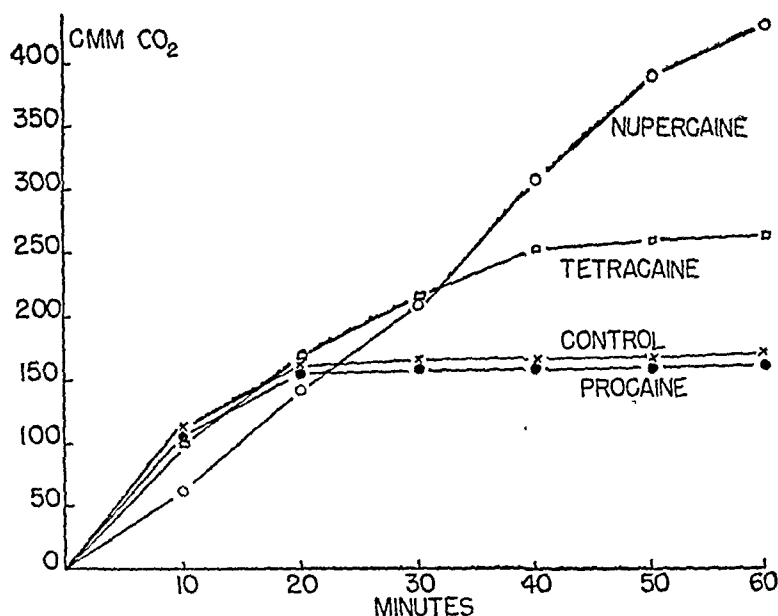


FIG. 3. EFFECT OF LOCAL ANESTHETICS ON ANAEROBIC GLYCOLYSIS IN BRAIN HOMOGENATES

Temperature, 36.3°C.; gas phase, 95 per cent nitrogen plus 5 per cent carbon dioxide; concentration of solutions: glucose, 0.028 *M*; hexose diphosphate-Mg, 0.0025 *M*; adenosine triphosphate, 0.0007 *M*; DPN, 0.0005 *M*; nicotinamide, 0.04 *M*;  $K_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4, 0.01 *M*;  $NaHCO_3$ , 0.048 *M*; drug, 0.005 *M*; final volume, 1.0 cc.

TABLE 1

*Effect of local anesthetics on the respiration of brain homogenates*

SUBSTRATE	CON- TROL	COCAINE		PROCAINE		METYCAINE		BUTACARINE		NUPERCARINE		TETRACARINE	
		Cmm. $O_2$	Per cent diff.	Cmm. $O_2$	Per cent diff.	Cmm. $O_2$	Per cent diff.	Cmm. $O_2$	Per cent diff.	Cmm. $O_2$	Per cent diff.	Cmm. $O_2$	Per cent diff.
	<i>cmm. <math>O_2</math></i>												
Glucose oxidation...	177	121	-32	139	-22	96	-46	65	-63	47	-74	64	-64
Succinate oxidation.	369	336	-9	329	-11	339	-8	183	-51	18	-95	206	-44
Ascorbate oxidation.	305	312	+2	280	-9	295	-4	192	-21	100	-68	195	-36
Glucose* anaerobic glycolysis.....	176	193	+10	170	-3	180	+2	180	+2	460	+161	262	+49

All experiments were of 60 minutes duration at 36.3° C. with a drug concentration of 0.005 *M*. Concentration of solutions in Warburg vessels same as those given in figs. 1 and 3.

\* Values for anaerobic glycolysis are in cmm.  $CO_2$ .

anaerobic reduction of methylene blue is shown in table 2. Procaine, metycaine and nupercaine greatly accelerated the reduction of methylene blue in the absence of added substrate. Slight acceleration is shown in the presence of succinate. Cocaine inhibited the reaction both in the absence and presence of succinate. Table 4 shows that nupercaine inhibits both the oxidation and reduction of cytochrome c.

TABLE 2

*The effect of local anesthetics on succinic dehydrogenase*

DRUG CONCENTRATION Molar	REDUCTION TIME IN MINUTES							
	No substrate		0.04 M sodium succinate					
	0.0	0.01	0.0	0.0025	0.005	0.01	0.02	0.04
Procaine	214	15	17	—	15	14	14	13
Cocaine	195	>270	21	24	24	27	34	40
Nupercaine	170	18	17	17	16	14	—	—

Except as indicated each Thunberg tube contained 1.0 cc. of 0.13 M sodium succinate, 1.0 cc. of 0.10 M phosphate buffer, pH 7.4, 1.0 cc. of 1.10.000 methylene blue, drug to give final concentration shown in table, water to 5 cc., 1.0 cc. of 1.10 brain homogenate in phosphate buffer in the side arm, temperature 37°C

TABLE 3

*Effect of nupercaine on lactic acid formation in brain homogenates*

Lactic acid in mgm./Warburg vessel/60 minutes

EXPT. NO.	CONTROL	0.005 M NUPERCANE
1	0.31	1.14
2	0.28	1.45
3	0.20	0.73

Solutions used and experimental conditions same as those given for anaerobic glycolysis in fig. 3

TABLE 4

*Effect of nupercaine and procaine on the oxidation and reduction of cytochrome c*

	CONTROL	DRUG	DIFF. per cent
Oxidation of $CyFe^{++}$ , Nupercaine, 0.0025 M	0.316	0.053	-83
Oxidation of $CyFe^{++}$ , Procaine 0.005 M	0.555	0.593	+6.9
Reduction of $CyFe^{+++}$ , Nupercaine 0.0025 M	0.120	0.025	-79
Reduction of $CyFe^{+++}$ , Procaine 0.005 M	0.126	0.100	-7.9

Control and drug values are in terms of  $\Delta \log CyFe^{++}$  for oxidation experiments and  $\Delta \log CyFe^{+++}$  for reduction experiments for 0-10 minute intervals. For oxidation experiments each vessel contained 0.02 M phosphate buffer, pH 7.0, drug to indicated concentration, 0.1 cc. of 1.200 brain homogenate, 0.00003 M  $CyFe^{++}$  previously reduced with hyposulfite added at zero time, total volume 3.0 cc. For reduction experiments each vessel contained 0.02 M phosphate buffer, pH 7.0, drug to indicated concentration, 0.001 M NaCN, 0.00003  $CyFe^{+++}$ , 0.1 cc. of 1.200 brain homogenate, 0.05 M succinate added at zero time. All experiments at room temperature.

The effect of the local anesthetics on anaerobic glycolysis is shown in fig. 3 and table 1. Tetracaine and nupercaine increase the amount of carbon dioxide liberated during the 60 minute experiment. When glycolysis is correlated with time, the activity with and without the drug is approximately the same for the first ten minutes. In the presence of nupercaine this rate is maintained for the 60 minute experiment whereas in the control vessel glycolysis rapidly diminishes after the first ten minutes. Thus the increase is not a stimulation



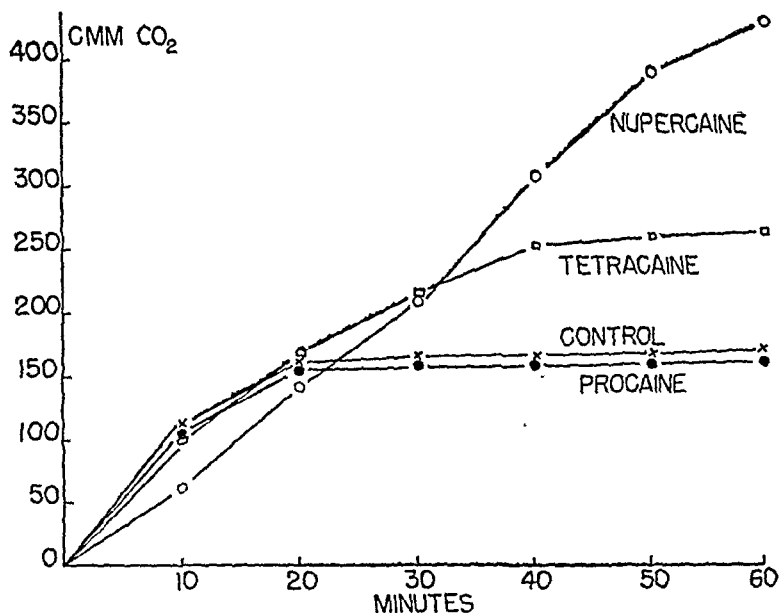


FIG. 3. EFFECT OF LOCAL ANESTHETICS ON ANAEROBIC GLYCOLYSIS IN BRAIN HOMOGENATES

Temperature, 36.3°C.; gas phase, 95 per cent nitrogen plus 5 per cent carbon dioxide; concentration of solutions: glucose, 0.028 *M*; hexose diphosphate-Mg, 0.0025 *M*; adenosine triphosphate, 0.0007 *M*; DPN, 0.0005 *M*; nicotinamide, 0.04 *M*;  $K_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4, 0.01 *M*;  $NaHCO_3$ , 0.048 *M*; drug, 0.005 *M*; final volume, 1.0 cc.

TABLE 1

*Effect of local anesthetics on the respiration of brain homogenates*

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		Cmm. O <sub>2</sub>	Per cent diff.	Cmm. O <sub>2</sub>	Per cent diff.	Cmm. O <sub>2</sub>	Per cent diff.	Cmm. O <sub>2</sub>	Per cent diff.	Cmm. O <sub>2</sub>	Per cent diff.	Cmm. O <sub>2</sub>	Per cent diff.
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\* Values for anaerobic glycolysis are in cmm. CO<sub>2</sub>.

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greater than that in the vessels with the drug for the first ten minutes of the experiment. Thus the increase appears to be due to a maintenance of the high original rate of glycolysis by some unexplained mechanism. As shown in table 5 a correlation can be made between the *in vitro* inhibition of carbohydrate oxidation observed in these experiments and the *in vivo* potency of the local anesthetics investigated. In both the comparative experiments at 0.005 *M* and in the determinations over a range of concentrations (fig. 2) cocaine, procaine and metycaine produce the least inhibition, tetracaine and butacaine are intermediate, and nupercaine gives the greatest inhibition. This is in close agreement with the *in vivo* potency of these anesthetics as determined in pharmacological experiments (16, 18) and by clinical experience (17).

The author wishes to thank Dr. C. L. Gemmill for his valuable suggestions in connection with this work and Miss Doris Haynes and Mrs R. F. Matthews for their technical aid in the experiments.

#### SUMMARY

Cocaine, procaine, metycaine, butacaine, tetracaine and nupercaine inhibit the oxidation of glucose, succinate and ascorbate but do not inhibit the anaerobic glycolysis of glucose by brain homogenates. The oxidation and reduction of cytochrome *c* is inhibited; the anaerobic reduction of methylene blue in the presence of succinate and homogenate is not inhibited. These results indicate the blockage of the enzymatic chain is occurring at the cytochrome *c*-cytochrome oxidase level or at some factor necessary for the reduction of cytochrome *c*.

There is a wide range in the degree of inhibition by the anesthetics at 0.005 *M* and over a range of concentrations. Cocaine, procaine and metycaine give the least inhibition; tetracaine and butacaine are next in order; and nupercaine produces the greatest inhibition. There is a correlation between the *in vitro* and *in vivo* order of inhibition of these drugs.

#### REFERENCES

1. QUASTEL, J. H., AND WHEATLEY, A. H. M.: *Proc. Roy. Soc. London*, B112: 60, 1932
2. JOWETT, M.: *J. Physiol.*, 92: 322, 1935
3. MICHAELIS, M., AND QUASTEL, J. H.: *Biochem. J.*, 35: 518, 1941.
4. FUHRMAN, F. A., AND FIELD, J.: *THIS JOURNAL*, 77: 392, 1913
5. GREIG, M. E., AND HOWELL, R. S.: *Arch. Biochem.*, 19: 441, 1915
6. NIWA, S.: *THIS JOURNAL*, 12: 323, 1919
7. SHERIF, M. A. F.: *THIS JOURNAL*, 38: 11, 1930
8. BALL, E. G., ANFINSEN, C. B., AND COOPER, O.: *J. Biol. Chem.*, 168: 257, 1947.
9. UTTER, M. F., WOOD, H. G., AND REINER, J. M.: *J. Biol. Chem.*, 161: 197, 1945
10. REINER, J. M.: *Arch. Biochem.*, 12: 327, 1917
11. SCHNEIDER, W. C., AND POTTER, V. R.: *J. Biol. Chem.*, 149: 217, 1913
12. EDWARDS, H. T.: *J. Biol. Chem.*, 125: 571, 1938
13. ALTSCHUL, A. M., ABRAMS, R., AND HOGNESS, T. R.: *J. Biol. Chem.*, 136: 777, 1940
14. POTTER, V. R., AND ELVEHJEM, C. A.: *J. Biol. Chem.*, 114: 195, 1936
15. WATTS, D. T.: *THIS JOURNAL*, 95: 117, 1919
16. HIRSCHFELDER, A. D., AND BIRTFER, R. N.: *Physiol. Rev.*, 12: 190, 1932
17. ADRIANI, J.: *The Pharmacology of Anesthetic Drugs*. Charles C. Thomas, Springfield, Ill., 1941
18. HAMILTON, H. S., WESTFALL, B. A., AND FERGUSON, J. K. W.: *THIS JOURNAL*, 94: 299, 1918

but a maintenance of the system's normal glycolysis. The protection of the anaerobic glycolytic enzyme system appeared to be of the same type as that obtained when nicotinamide is added to tissue homogenates to prevent the autolytic destruction of DPN by DPNase (14). Several experiments were conducted in which the DPN, nicotinamide and nupercaine concentrations were varied in an attempt to show that nupercaine was inhibiting DPNase or some other autolytic enzyme. Such protection could not be demonstrated.

Lactic acid determinations made on the contents of Warburg vessels after anaerobic glycolysis experiments are given in table 3. These data show that

TABLE 5

*Comparison of the anesthetic potency of local anesthetics with the inhibition of succinate oxidation*

DRUG	RELATIVE POTENCY	RELATIVE TOXICITY	SUCCINATE OXIDATION PER CENT INHIBITION BY 0.005 M DRUG
Cocaine.....	0.88	2.28	-9
Procaine.....	1.00	1.00	-11
Metycaine.....	2.90	1.12	-8
Butacaine.....	5.30	2.01	-51
Tetracaine.....	9.00	1.80	-44
Nupercaine.....	25.90	4.03	-95

The data on relative anesthetic potency and toxicity are from ref. 18 and based on procaine = 1. Potency was determined by intradermal injection and toxicity by intraperitoneal injection of guinea pigs.

there is increased production of lactic acid in the presence of nupercaine. The possibility that nupercaine was breaking down or reacting chemically to release carbon dioxide from the reaction mixture was investigated. Dilute hydrochloric acid was accurately added to the reaction mixture from the side arm. Equal amounts of carbon dioxide were liberated from the buffer in the presence and absence of nupercaine.

**DISCUSSION.** The local anesthetics investigated in these experiments showed selective inhibition of respiration of brain homogenates in that the oxidation of glucose, succinate and ascorbate was inhibited whereas the anaerobic glycolysis of glucose was never inhibited. This indicates the inhibition occurs in the oxidative enzyme chain. It is of interest that the oxidation of ascorbate which is catalyzed by cytochrome c-cytochrome oxidase is inhibited. That inhibition is occurring at this level is further supported by the fact that the oxidation of reduced cytochrome c by cytochrome oxidase is inhibited by nupercaine. Nupercaine likewise inhibits the reduction of cytochrome c by the homogenate with succinate as substrate. These observations indicate the effect is on the cytochrome c-cytochrome oxidase or some factor necessary for the reduction of the cytochrome because nupercaine does not inhibit succinic dehydrogenase as determined by the Thunberg method. The increased anaerobic glycolysis in the presence of nupercaine as determined by carbon dioxide liberation cannot be considered an acceleration of glycolysis because the control rate is equal to or

greater than that in the vessels with the drug for the first ten minutes of the experiment. Thus the increase appears to be due to a maintenance of the high original rate of glycolysis by some unexplained mechanism. As shown in table 5 a correlation can be made between the *in vitro* inhibition of carbohydrate oxidation observed in these experiments and the *in vivo* potency of the local anesthetics investigated. In both the comparative experiments at 0.005 *M* and in the determinations over a range of concentrations (fig. 2) cocaine, procaine and metycaine produce the least inhibition, tetracaine and butacaine are intermediate, and nupercaine gives the greatest inhibition. This is in close agreement with the *in vivo* potency of these anesthetics as determined in pharmacological experiments (16, 18) and by clinical experience (17).

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Cocaine, procaine, metycaine, butacaine, tetracaine and nupercaine inhibit the oxidation of glucose, succinate and ascorbate but do not inhibit the anaerobic glycolysis of glucose by brain homogenates. The oxidation and reduction of cytochrome *c* is inhibited, the anaerobic reduction of methylene blue in the presence of succinate and homogenate is not inhibited. These results indicate the blockage of the enzymatic chain is occurring at the cytochrome *c* cytochrome oxidase level or at some factor necessary for the reduction of cytochrome *c*.

There is a wide range in the degree of inhibition by the anesthetics at 0.005 *M* and over a range of concentrations. Cocaine, procaine and metycaine give the least inhibition, tetracaine and butacaine are next in order, and nupercaine produces the greatest inhibition. There is a correlation between the *in vitro* and *in vivo* order of inhibition of these drugs.

#### REFERENCES

1. QUASTEL J. H. AND WHEATLEY A. H. M. *Proc Roy Soc London*, B112 60, 1932
2. JOWETT, M. *J Physiol*, 92 322 1938
3. MICHAELIS M., AND QUASTEL J. H. *Biochem J*, 35 518, 1941
4. FUHRMAN, F. A., AND FIFID, J. *THIS JOURNAL*, 77 392, 1913
5. GREIG M. E. AND HOWELL R. S. *Arch Biochem*, 19 411 1918
6. NIWA S. *THIS JOURNAL* 12 323 1919
7. SHERIF, M. A. F. *THIS JOURNAL* 38 11, 1930
8. BALL E. G., ANFINSEN C. B., AND COOPER O. *J Biol Chem*, 168 257, 1917
9. UTTER M. F., WOOD H. G. AND REINER J. M. *J Biol Chem*, 161 107, 1915
10. REINER J. M. *Arch Biochem* 12 327, 1917
11. SCHNEIDER W. C., AND POTTER V. R. *J Biol Chem*, 149 217, 1913
12. EDWARDS, H. T. *J Biol Chem* 125 571, 1939
13. ALTSCHUL A. M., ABRAMS, R. AND HOGNESS T. R. *J Biol Chem* 136 777, 1940
14. POTTER, V. R. AND ELVEHJEM C. A. *J Biol Chem* 114 495, 1936
15. WATTS D. T. *THIS JOURNAL* 95 117, 1919
16. HIRSCHFELDER A. D., AND BIETTER R. N. *Physiol Rev*, 12 100 1932
17. ADRIANI, J. *The Pharmacology of Anesthetic Drugs*. Charles C. Thomas Springfield, Ill., 1941
18. HAMILTON H. S., WESTFALL, B. A., AND FERGUSON, J. K. W. *THIS JOURNAL* 94 299, 1918

# THE ACTION OF TRIPELENNAMINE ON HYALURONIDASE IN THE ALBINO RAT

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Tripelennamine (Pyribenzamine) (*N'*-pyridyl-*N'*-benzyl-*N*-dimethylethylene diamine hydrochloride) is an antiallergic and antihistaminic drug, that has wide therapeutic applications. Its principle pharmacologic property is the antagonism of histamine, although it is not effective against the mydriasis and hypotension produced by histamine in the cat (1). Pyribenzamine also has other effects; it is an antispasmodic and local anesthetic (2). Sherrod *et al.* (3) found that large doses of the drug potentiated the pressor action of epinephrine and stimulated the uterus and duodenum in dogs.

Mayer and Kull (2) reported that in albino rats, Pyribenzamine strongly reduced the extent of the normal dermal spreading of injected India ink, as well as the spread produced in the presence of hyaluronidase. However, it has been noted (4) that antihistaminic agents did not diminish the infiltration of intravenously administered trypan blue into areas of increased capillary permeability, except when histamine was demonstrated to be the cause of the increased permeability. No modification of the trypan blue test occurred where the locally acting substance was horse serum, trypsin, snake venom or staphylococcus toxin. Guerra (5) has demonstrated that the administration of sodium salicylate to rabbits suppressed the dermal spreading effect of hyaluronidase. It was suggested by Swyer (6) that this inhibition was due to an antihistaminic and not an antihyaluronidase action of the salicylates.

A technique has been described (7) in which the *in vivo* activity of hyaluronidase may be tested in the albino rat. When a sufficient amount of hyaluronidase is administered intravenously, diffusion of fluid across the capillary membrane occurs, accompanied by a rise in the blood hematocrit. Under given conditions, a standard amount of enzyme produces a predictable rise in the hematocrit. If, following the administration of a substance, the hemoconcentration and edema do not occur in response to the injection of the enzyme, that substance may be considered a hyaluronidase antagonist.

Because of the role that histamine is considered to play in certain conditions involving fluid exchange across the capillary membrane, and because it has been implicated by some in the action of hyaluronidase in the body, the study of the efficacy of Pyribenzamine to protect against the action of hyaluronidase was undertaken.

**METHODS AND MATERIALS.** One-hundred and fifty-nine male albino rats of the Sherman strain, weighing 160-230 gm., were fed Purina laboratory chow and water *ad lib*. The hyaluronidase was prepared according to the method of Freeman *et al.* (8). The enzyme was assayed (9) and measured 3000 turbidity reducing units (T.R.U.) per mgm. of nitrogen.

Pyribenzamine was dissolved in 0.85 per cent NaCl solution, so that 1 cc contained 20 mgm Pyribenzamine. A dose of 7.5 mgm of the drug per 100 gm of body weight was administered subcutaneously to 30 rats. Twenty minutes later, the animals were anesthetized with ether and hyaluronidase was injected intravenously, ten rats each were given 2500 T R U, 3750 T R U or 5000 T R U. Forty rats received no pretreatment with Pyribenzamine, of these, twenty animals were given 2500 T R U, and ten rats each were administered 3750 or 5000 T R U hyaluronidase intravenously. At the end of 30 minutes, all were bled from the aorta and sacrificed. An additional twenty rats were injected subcutaneously with Pyribenzamine, 7.5 mgm/100 gm. One half of this group was bled twenty minutes and the remainder 50 minutes later. Another group of twenty normal rats were bled and sacrificed. The hematocrits of all were determined by centrifugation at 3000 r p m for 30 minutes.

The duration of the *in vivo* inhibitory action of 7.5 mgm Pyribenzamine/100 gm on 2500 T R U hyaluronidase was studied. The enzyme was administered intravenously to groups of five rats at 0, 1/3, 2, 8 and 24 hours following the Pyribenzamine. The animals were bled 30 minutes later and the hematocrits determined in the usual manner.

The influence of the dose of Pyribenzamine on the hyaluronidase inhibition was investigated. Pyribenzamine in doses of 7.5, 4.0, 0.8, 0.1, 0.1 and 0.05 mgm/100 gm was injected subcutaneously, 2500 T R U hyaluronidase was given intravenously twenty minutes later. One half hour following this, aortic blood was withdrawn and the hematocrits were measured.

Diphenhydramine (Benadryl) (beta-dimethylamino ethyl benzhydryl ether hydrochloride), another antihistaminic agent, was tested for its ability to antagonize hyaluronidase *in vivo*. It was given subcutaneously to five rats 7.5 mgm/100 gm. The test amount of hyaluronidase 2000 units, was injected twenty minutes later, and the hematocrits of the blood obtained at 30 minutes were determined.

The ability of Pyribenzamine to inhibit hyaluronidase under *in vitro* conditions was tested. Pyribenzamine in saline solution, was incubated at room temperature with hyaluronidase in phosphate saline buffer at pH 5 for 30 minutes. At the end of that time, the enzyme was assayed in the conventional manner (9). The influence of pH, buffer solutions, temperature, enzyme and Pyribenzamine concentrations was tested.

As a further aid in the elucidation of the mechanism of action of Pyribenzamine on hyaluronidase the effect of this drug on the blood inhibitors of hyaluronidase was studied. A non specific inhibitor is present in blood plasma and can be measured by a turbidimetric method described by Dorfman (10). The titer of inhibitors in the sera of thirteen normal male rats was measured, as well as the level of inhibitors for eighteen male rats that received 7.5 mgm Pyribenzamine/100 gm.

**RESULTS** The animals that received 2500-5000 T R U hyaluronidase alone exhibited the changes described previously (7). Edema of the extremities and face appeared within five to fifteen minutes, and became maximal at 30 minutes, at which time the rats were sacrificed. The mean hematocrits for these groups were 60.0, 61.0 and 62.9 per cent (figure 1). Of the group that was pretreated with Pyribenzamine and then received 2500 units hyaluronidase, none developed edema. The range of hematocrits was normal and the mean value, 45.5 per cent, was not significantly altered. Of the rats that received Pyribenzamine and then 3750 units hyaluronidase, two had no change at all in the hematocrit, two to four had a moderate rise and the remainder rose to the expected levels. The mean hematocrit, 56.3 per cent was significantly less than the 61.0 per cent in the untreated group. A similar partial inhibition occurred with those pretreated animals that received 5000 units hyaluronidase. The

range (42–50 per cent) and the mean hematocrit (46.0 per cent) obtained for the twenty normal rats was almost identical for that of a group of 150 normal male rats in this laboratory. Pyribenzamine alone caused no significant alteration of the hematocrits of twenty rats.

The time interval between the administration of Pyribenzamine and the test dose of the enzyme influenced the results (table 1). When they were injected simultaneously, a wide range of hematocrits was obtained, depending on the

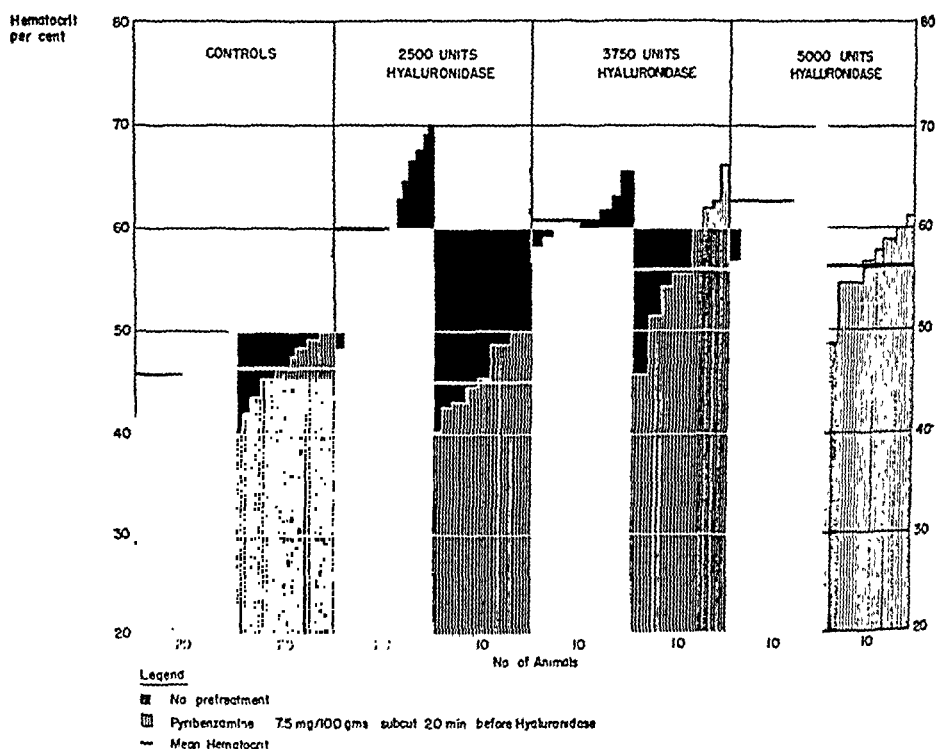


FIG. 1. IN VIVO INHIBITION OF HYALURONIDASE BY PYRIBENZAMINE

The modification by Pyribenzamine of the hematocrit response of the albino rat following the intravenous injection of hyaluronidase.

rate of absorption of Pyribenzamine from the subcutaneous region. The mean value (55.2 per cent) was significantly lower than the expected value (60.0 per cent), and was evidence of a partial antagonism. At twenty minutes and two hours, complete inhibition occurred. After eight hours, the Pyribenzamine had lost very little of its effect; the mean hematocrit one-half hour following the test dose of enzyme was 49.8 per cent. After 24 hours, Pyribenzamine no longer modified the action of hyaluronidase.

Variation of the dose of Pyribenzamine disclosed (table 2) that 4 or more mgm. Pyribenzamine per 100 gm. produced complete inhibition; 0.4–0.8 mgm./100 gm. resulted in partial inhibition and 0.1 or less mgm./100 gm. caused little or no modification of the action of hyaluronidase.

Benadryl was approximately as active as Pyribenzamine (table 3) in its inhibitory action on hyaluronidase

Pyribenzamine failed to inhibit hyaluronidase *in vitro*. The concentration of Pyribenzamine was varied from 0.01 to 2.5 mgm /cc and the hyaluronidase

TABLE 1

*Duration of in vivo inhibition of 2500 units hyaluronidase following administration of Pyribenzamine*

TIME OF ADMINISTRATION OF PYRIBENZAMINE PRIOR TO HYALURONIDASE*	NO. OF ANIMALS	MEAN HEMATOCRIT
hrs		per cent
0	5	55.2
1	10	45.5
2	5	41.2
8	1	49.8
24	5	60.5

\* Pyribenzamine injected subcutaneously 7.5 mgm /100 gm

TABLE 2

*Effect of dosage of Pyribenzamine on in vivo inhibition of 2500 units hyaluronidase*

DOSAGE	NO. OF ANIMALS	MEAN HEMATOCRIT
mgm /100 gm		per cent
7.5	10	45.5
1.0	5	46.0
0.8	5	53.8
0.4	5	50.8
0.1	5	57.6
0.05	5	58.6

Hyaluronidase injected 20 minutes after administration of Pyribenzamine

TABLE 3

*In vivo inhibition of hyaluronidase by Pyribenzamine and Benadryl*

DRUG	AMOUNT OF HYALURONIDASE	NO. OF ANIMALS	MEAN HEMATOCRIT
	TRU		per cent
Pyribenzamine*	2500	10	45.5
Benadryl*	2500	5	48.2
None	2500	20	60.0
None	0	20	46.0

\* Drugs administered subcutaneously 20 minutes prior to the hyaluronidase. Dosage 7.5 mgm /100 gm

from 6 to 330 TRU /cc. This reaction was tested at pH 5 and 7, at 20.0°C and 37.5°C, and in borate buffer and in phosphate saline buffer.

The mean of the blood plasma inhibitors of thirteen normal male rats was  $75.1 \pm 4.3$  units per cc. Eighteen rats received 7.5 mgm /100 gm of Pyribenzamine subcutaneously and the mean plasma inhibitor level was  $63.8 \pm 6.6$  units per cc.



The difference, 11.3 units/cc., was within the experimental error and was not significant.

The enzyme used in these experiments was analyzed (11) for histamine<sup>1</sup>; none was present.

**Discussion.** Pyribenzamine inhibited the "hematocrit and fluid diffusion" effects of hyaluronidase in the albino rat. The action of 2500 T.R.U. of the enzyme was completely antagonized and partial modification of the effects of 5000 T.R.U. was obtained. When Pyribenzamine was administered in doses comparable to those given to humans, only partial inhibition of the hyaluronidase was elicited. However, adequate doses of the drug were effective up to eight hours following its administration. Benadryl, another antihistaminic compound with a different chemical structure, was equally active in antagonizing hyaluronidase *in vivo*. These experiments confirm those of Mayer and Kull, in which they described inhibition of the skin diffusing action of hyaluronidase in the rat.

Some of the factors that might be concerned with this *in vivo* property of Pyribenzamine have been investigated. Since Pyribenzamine and Benadryl are primarily antihistaminics, the role of histamine must be considered. Swyer's suggestion that histamine, that might have been present in testicular extract, was responsible for the spreading action of hyaluronidase, was not confirmed; no histamine was demonstrated in the hyaluronidase preparations. The intravenous administration of 1.0 mgm. histamine phosphate failed to produce comparable effects in the albino rat (7). In addition, neither the "fluid diffusion and hematocrit" effects nor anaphylaxis resulted from the intravenous administration of large amounts of hyaluronidase to the guinea-pig, an animal particularly sensitive to the action of histamine. However, histamine or a histamine-like substance may be elaborated by the rat in response to the injection of hyaluronidase, and the Pyribenzamine may exert its blocking action at this site. The other possibilities to be considered are that a yet unknown intermediate or group of intermediates are antagonized by the antihistaminics, or that Pyribenzamine is activated by some substance in the body so that becomes a true anti-hyaluronidase substance.

*In vitro*, Pyribenzamine did not inhibit hyaluronidase. The level of blood inhibitors was not increased by this drug, nor was there any demonstrable alteration of the hemodynamics of the rat. The exact mechanism of inhibition is still obscure.

#### SUMMARY AND CONCLUSIONS

1. Tripeleennamine (Pyribenzamine) (N'-pyridyl-N'-benzyl-N-dimethylethylene diamine hydrochloride) inhibited the "hematocrit and fluid diffusion" effects of intravenously administered hyaluronidase in the albino rat.

2. *In vitro*, hyaluronidase was not antagonized by Pyribenzamine.

3. The level of non-specific plasma inhibitors of hyaluronidase did not rise following the administration of Pyribenzamine.

<sup>1</sup> The authors are grateful to Drs. Sanford M. Rosenthal and R. C. Millikan, National Institute of Health, Bethesda, Md., who performed the assay.

4. The possible role of the anti-histaminic drugs in hyaluronidase inhibition was discussed.

## REFERENCES

- 1 YONAMAN, F. F., CHIFFS, D., MATHESON, D., AND HANSEN, N. THIS JOURNAL, 87: 256, 1946
- 2 MAYR, R. L., AND KELL, F. C. Proc Soc Exper Biol & Med, 66: 392, 1947
- 3 SHERRER, T. R., SCHLOFFER, H. F., AND LOW, E. R. Fed Proc, 6: 202, 1946
- 4 LAST, M. R., AND LOW, E. R. THIS JOURNAL, 69: 81, 1947
- 5 GUHRA, F. THIS JOURNAL, 87: 193, 1946
- 6 SWYER, G. I. M. Biochem J, 42: 28, 1948.
- 7 ELSTER, S. K., GREFMAN, M. E., AND ANDERSON, P.: J Lab & Clin Med, in press, 1949
- 8 GREFMAN, M. E., ANDERSON, P., OBERG, M., AND DORFMAN, A. in press, 1949
- 9 DORFMAN, A., AND OTT, M. L. J Biol Chem, 172: 367, 1948
- 10 DORFMAN, A., OTT, M. L., AND WHITNEY, R. J Biol Chem, 174: 621, 1948
- 11 ROSENTHAL, S. M., AND TABOR, H. THIS JOURNAL, 92: 125, 1948

# COMPARATIVE SPASMOLYTIC ACTIVITIES OF CERTAIN BENZHYDRYL ALKAMINE ETHERS AND RELATED COMPOUNDS

GORDON A. ALLES AND MILDRED A. REDEMANN

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The studies of Loew, Kaiser and Moore (1) on the effectiveness of some seventeen various benzhydryl alkamine ethers in preventing fatal histamine-induced bronchoconstriction in guinea pigs, established the general range of structure exhibiting anti-histamine activity in this type of compound. The most active of the compounds they studied was the benzhydryl  $\beta$ -dimethyl-aminoethyl ether, later to be known in the form of its hydrochloride under the trade name of Benadryl. While a relatively large number of compounds were studied, consideration of the structures in detail shows the  $\beta$ -methyl- $\beta$ -morpholinopropyl ether to be the only branched chain aminoalkyl ether studied, and the  $\gamma$ -diethylaminopropyl and  $\gamma$ -morpholinopropyl ethers the only  $\gamma$ -aminopropyl ethers studied. Inasmuch as the morpholino and diethylamino groups in compounds were found to be distinctly less active than a corresponding dimethylamino group, a study of the effect of methyl substitution of the ethyl group in dimethylaminoethyl benzhydryl ether was indicated, and also a study of the benzhydryl  $\gamma$ -dimethylaminopropyl ether.

Four mono-substituted derivatives of benzhydrylamine were also included in these earlier reported studies, but dimethylaminoethyl compounds were not included. Inasmuch as mono-substituted amines correspond to both alcohols and ethers in the nitrogen system of compounds, it was necessary to prepare the dimethylaminoethyl derivative of benzhydryl methylamine in order to afford a true comparison between benzhydryl oxygen and nitrogen ethers (see Franklin (2)).

The earlier studies of Lehmann and Knoefel (3) of the spasmolytic action of a large number of aminoalkanol esters of 9,10-dihydroanthracene carboxylic acid and related compounds had shown that esters of this type might also have very high anti-histamine activities as well as considerable anti-acetylcholine activities. Accordingly, our study was extended in the benzhydryl series of compounds to esters corresponding closely in structure to the ethers that had been shown to be most active with regard to antagonizing histamine. This extension of our study led to compounds like and including the diphenylacetate ester of diethylaminoethanol, which is known under the trade name of Trasentine. This compound is clinically notable for its anti-acetylcholine activities, and this series of our compounds was also comparatively valued for this kind of activity.

The compounds used were synthesized in our laboratories by C. Ernst Redemann and Burnett B. Wisegarver, their identity being concluded from the methods of synthesis used, together with precise analyses of nitrogen or halide content, or both.

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TABLE 1

*Average reduction of guinea pig ileum response to  $10^{-6}$  molar histamine*  
(Number of trials averaged)

CHEMICAL COMPOUND	FINAL CONCENTRATION		
	$10^{-7}$ M	$5 \times 10^{-7}$ M	$10^{-6}$ M
#1 $\beta$ -Diphenylmethoxy-ethyl dimethylamine (Benadryl) HCl MW 292	44% (5)	88% (3)	
#2 $\alpha$ -Methyl- $\beta$ -diphenylmethoxy-ethyl dimethylamine HCl MW 306	15% (6)	20% (4)	
#3 $\alpha, \alpha$ -Dimethyl- $\beta$ -diphenylmethoxy-ethyl dimethyl- amine HCl MW 320	13% (5)	46% (5)	
#1 $\beta$ -Diphenylmethoxy-ethyl dimethylamine (Benadryl) HCl MW 292		81% (4)	
#4 $\beta$ -Methyl- $\beta$ -diphenylmethoxy-ethyl dimethylamine HCl MW 306	1% (2)	11% (4)	
#5 $\beta, \beta$ -Dimethyl- $\beta$ -diphenylmethoxy-ethyl dimethyl- amine HCl MW 320	2% (2)	3% (4)	
#1 $\beta$ -Diphenylmethoxy-ethyl dimethylamine (Benadryl) HCl MW 292	49% (3)	79% (10)	
#6 $\gamma$ -Diphenylmethoxy-propyl dimethylamine acid succinate MW 388	12% (4)	61% (9)	
#1 $\beta$ -Diphenylmethoxy-ethyl dimethylamine (Benadryl) HCl MW 292	63% (2)	77% (3)	
#7 $\beta$ -Triphenylmethoxy-ethyl dimethylamine HCl MW 368	0% (1)	10% (6)	
#1 $\beta$ -Diphenylmethoxy-ethyl diamethylamine (Benadryl) HCl MW 292		53% (2)	92% (7)
#8 $\beta$ -Diphenylmethylamino-ethyl dimethylamine diHCl MW 327		3% (3)	40% (6)
#9 $\beta$ -Diphenylmethyl-methylamino-ethyl dimethylamine diHCl MW 341		5% (2)	3% (4)
#10 $\beta$ -Diphenylmethylamino-ethyl trimethylammonium methosulfate MW 380		0% (1)	26% (6)

as when the link was of an alcohol-ether of the nitrogen system. The quaternary ammonium compound #10 was of closely the same activity as the tertiary amine compound #8, fitting in with the observations of Loew, MacMillan and

trials) reduction under the same conditions. This fits in with the observations of Winder, Kaiser, Anderson and Glassco (5) that the conversion of Benadryl into the corresponding quaternary ammonium does not notably affect anti-histamine activity, but does appreciably increase anti-acetylcholine activity under the test conditions.

#### SUMMARY

1. The anti-histamine activities of a series of ethyl substituted dimethylaminoethyl benzhydryl ethers were evaluated and all found to be less active than the unsubstituted dimethylaminoethyl benzhydryl ether (Benadryl).

2. Certain corresponding derivatives of benzhydryl amine and methylamine were also studied and were found to be less active than the oxygen ethers in both their anti-histamine and anti-acetylcholine activities.

3. Dimethylaminoethanol benzhydrylacyl ester and dimethylalkylcarboxyl benzhydryl esters, closely corresponding in molecular size to the more active dimethylaminoalkyl benzhydryl ethers, were studied and found to be less active in their anti-histamine activities, and no more active in their anti-acetylcholine activities.

#### REFERENCES

1. LOEW, E. R., KAISER, M. E., AND MOORE, V.: *THIS JOURNAL*, 83: 120, 1945.
2. FRANKLIN, E. C.: *The Nitrogen System of Compounds*, Reinhold Publishing Corp., New York, 1935.
3. LEHMANN, G., AND KNOEFEL, P. K.: *THIS JOURNAL*, 80: 335, 1944.
4. LOEW, E. R., MACMILLAN, R., AND KAISER, M. E.: *THIS JOURNAL*, 86: 229, 1946.
5. WINDER, C. V., KAISER, M. E., ANDERSON, M. N., AND GLASSCO, E. M.: *THIS JOURNAL*, 87: 120, 1946.

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5. WINDER, C. V., KAISER, M. E., ANDERSON, M. N., AND GLASSCO, E. M.: *THIS JOURNAL*, 87: 120, 1946.

# CARDIO VASCULAR EFFECTS OF TWO ALIPHATIC AMINES AND OF EPHEDRINE<sup>1</sup>

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Two aliphatic amines, whose cardio vascular effects have been described in previous reports (1-7) have been subjected to further experimental study. In the experiments reported here, these amines, 2 methylamino 6 hydroxy 6 methyl heptane (EA 83) (Aranthol) and 6 methylamino 2 methyl 2 heptene (Octin) were further characterized by determinations involving contractile force changes of a section of the right ventricle, ECG recordings in unanesthetized dogs, isolated rabbit heart preparations, local vasoconstrictor experiments and a limited number of observations in patients at minimal dose ranges. The first of these compounds (Aranthol) was examined in most detail and, in some instances, direct experimental comparisons were made with ephedrine.

**EXPERIMENTAL** Contractile force changes of a section of the right ventricle were determined by procedures previously described (5). More recently, there has been a description of the physiologic factors influencing results obtained by this method (8). The method, as used here, involves the use of dogs in open chest preparations with simultaneous recording or determination of arterial pressure, heart rate and venous pressure, the latter being determined by a manometer containing heparinized saline and connected directly to a cannula opening either into the innominate vein or the superior vena cava. ECG recordings were all made from Lead II. Observations with isolated rabbit heart preparations were made with an apparatus designed to drain away the flow obtained from a light aluminum tube inserted into the left ventricle. This flow was recorded in addition to recordings of the perfusion flow collected in the usual way. The flow in each case was recorded drop wise by electronically actuated circuits. It has later been found desirable to insert a cog wheel counting arrangement in the circuit to limit recording to every tenth drop in the case of the flow ordinarily considered to represent coronary flow. Unless otherwise indicated, the amines were used in the form of their hydrochloride salts.

**RESULTS** *Experiments determining influence on contractile force of a section of the right ventricle* These experiments were designed to determine (1) the average dose producing an increase in contractile force of about 30 per cent (arbitrarily designated as the "stimulant dose"), (2) the average total dose which given in various installments produces a distinct depression of contractile force (arbitrarily designated as the "reversal dose"), (3) the direct comparison of "reversal doses" for two amines in the same experiment, (4) the approximate period at which a high level of increased contractile force can be maintained by the use of such amines.

Conclusions are based on previously reported experiments under the same conditions (4, 5), and, additionally, on 17 experiments subsequently conducted

<sup>1</sup> Preliminary abstract report in Fed. Proc., 6, 380, 1947.

<sup>2</sup> This study was supported in part by funds from the Billhuber Knoll Corporation, Orange, N. J.



Results are partly summarized in table 1. In these particular experiments, changes in heart size were not found to be of sufficient magnitude to warrant calculation of the corrections which might have been introduced to compensate for variations in heart size (8).

In determining the "stimulant doses" of table 1, it must be recognized that even with these small doses there is decreasing response with repeated injections. There is not, however, sufficiently rapid decrease to prevent a considerable number of comparative determinations at this low dose range in the same experiment. The figures for the "stimulant doses" refer to initial doses. Further, in the experiments determining "reversal doses" it must be recognized that at this higher dose range, these amines commonly produce varying degrees of initial, temporary depression followed by stimulation. In calculating the results, only the more prolonged stimulant phase was considered. The data in table 1 did not include experiments in which the amines were alternated at high dose ranges.

These data can be taken to mean that commonly ephedrine, when given in various installment schedules, will produce depression of cardiac contractility when the total dose reaches a figure about one hundred times greater than the

TABLE 1

AMINE SALT	AV. "STIMULANT DOSE"	AV. "REVERSAL DOSE"	"REVERSAL DOSE" "STIMULANT DOSE"
	mgm./kgm.	mgm./kgm.	
Ephedrine HCl.....	0.10	10.0	100
Octin HCl.....	0.15	13.5	90
Aranthol HCl.....	0.70	400.0	570

"stimulant dose." Likewise, Aranthol can be given by installments until the total dose is about five hundred and seventy times the "stimulant dose" before producing depression of cardiac contractility. Although these figures are considerably influenced by the size and frequency of the installments, they can be taken here as being generally representative of the relation between these drugs when compared on the basis of a variety of installment schedules.

*The direct comparison of "reversal doses" for two amines in the same experiment.* In a different type of experiment, Aranthol and ephedrine were alternated at high dose ranges, as illustrated in fig. 1. The "stimulant doses" were first determined in the same experiment and then increasing multiples of these doses were given. In the experiment illustrated, the "stimulant dose" with Aranthol was found to be about ten times greater than the comparable dose of ephedrine. Increasing doses of the two drugs were then given, the Aranthol doses being ten times greater than the ephedrine doses with which they were alternated. As can be seen from the figure, ephedrine produced distinct depression of the contractile force with doses approximately ten times those of the original "stimulant dose," while Aranthol continued to produce predominantly stimulant effects with doses approximately 160 times its original "stimulant dose".

Determinations of "reversal dose" indicate both the phylogenetic tendencies and limitation of the drug as a cardiac stimulant but need not necessarily give an index of the total dose necessary to stop the heart in this type of preparation. For instance, on occasion, at certain dose levels (in the range of 10 mgm /kgm ), ephedrine caused reversal of the typical cardiac stimulant action in the first few doses administered, yet the heart survived many subsequent doses of the same amount. In three instances of this sort, relatively tremendous total doses were given before stopping the heart. In the most extreme case, twenty doses of ephedrine 10 mgm /kgm, followed by eleven doses of 20 mgm /kgm were administered before stopping the heart. This amounted to a total of 1200

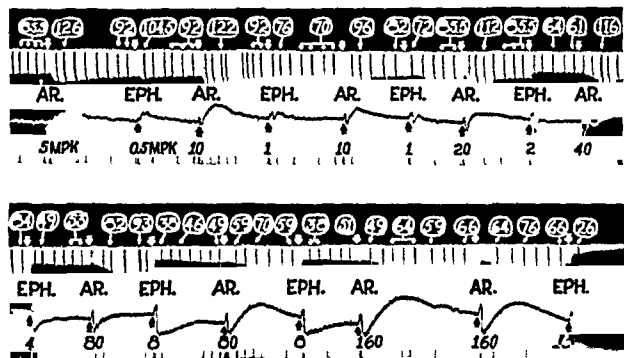


FIG. 1. Open chest dog preparation under pentobarbital barbital anesthesia with bilateral vago sympathetomy. Upper record —myocardiograph lever tracing. Circled figures —contractile force (in gms) of a section of the right ventricle. This may be referred to as isometric systolic tension (I.S.T.). Middle record —arterial pressure. Initial level in first section of this figure is about 90 mm Hg. Lowest record —time in minutes, at zero base line for arterial pressure. Ephedrine and Aranthol hydrochlorides were given intravenously, the doses of which are recorded in the lowest set of figures on the basis of mgm /kgm. This part of tracing was preceded by a series of comparative tests (17) in which Aranthol when given in 10 times greater doses was shown to be approximately equivalent to ephedrine in ability to increase contractile force.

"stimulant doses" during a period of five hours. In two other similar experiments, total doses in the range of 3500 "stimulant doses" were required. No such extreme amounts, in terms of "stimulant doses", were survived in the experiments with Aranthol, although not many experiments were conducted at this particular level of installment dosage.

On the other hand, when installments of two hundred "stimulant doses" were given ephedrine proved to be relatively more toxic. In two experiments, only two such injections (each about 20 mgm /kgm ) with ephedrine were necessary to stop the heart, whereas in four other experiments with Aranthol, three, five, eight, and eleven such injections (each about 200 mgm /kgm ) were survived.

*Determinations of the period during which a high level of increased contractile force can be maintained.* Two experiments of this sort with Aranthal are illustrated in figs. 2 and 3. In the first, a series of intravenous injections were given and in the second, one large subcutaneous injection was given after which subsequent injections were without important influence. From these and other experiments, it was concluded that with selected doses of Aranthal, the contractile force of the heart can be maintained for three to four hours at a level

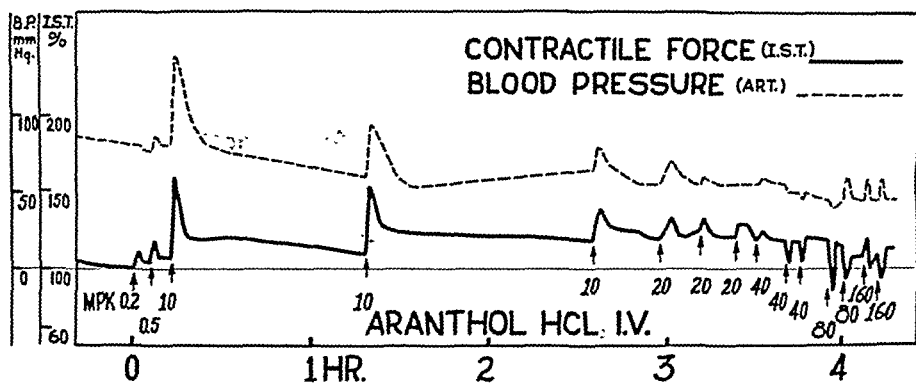


FIG. 2. Schematized record of changes in contractile force (expressed as percentage of that in the control period) and arterial pressure (in mm. Hg.). Same experimental conditions as in fig. 1. Aranthal HCl was given intravenously in doses indicated by figures below arrows (doses on basis of mgm./kgm.).

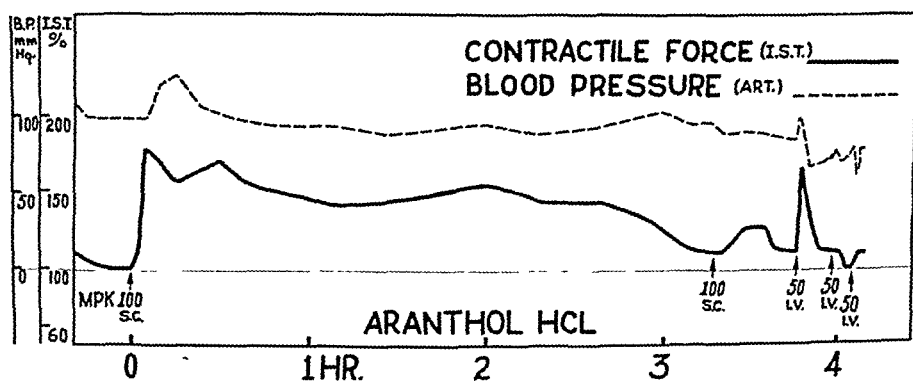


FIG. 3. Same recording and conditions as in fig. 2. Two doses of Aranthal HCl 100 mgm./kgm. subcutaneously were followed by three doses of 50 mgm./kgm. intravenously.

about 40 per cent greater than that of the control period. Higher levels (in the range of 100 per cent increase) can be obtained but will not ordinarily be sustained for this period of time.

These experiments demonstrate that when Aranthal is administered until the heart is no longer capable of further stimulation with this drug, the heart then becomes refractory and repetition of relatively large doses does not seriously depress the heart. This latter feature constitutes one of the major distinctions of this drug as contrasted with Octin or ephedrine.

*Concomitant effects* During all these experiments the responses in arterial pressure, which were determined simultaneously, indicated that Aranthal produced a somewhat lesser degree of hypertension than ephedrine when given in comparable myocardial stimulant doses. Also arterial pressure responses with Aranthal were more subject to tachyphylaxis than were responses of cardiac contractile force. With multiples of two hundred times the stimulant dose, Aranthal frequently produced a marked fall in arterial pressure while at the same time producing an increase in contractile force. With Aranthal, the venous pressure generally decreased slightly as the stimulant effect of the amine began, and then increased about 5 to 15 mm. water pressure as the stimulant action became fully established. Return to control level took place as the heart stimulant action diminished. The heart rate in these vagotomized animals was usually increased about 20 per cent when other stimulant effects were manifested.

*Electrocardiographic effects* Although premature beats and irregular rhythms were uncommon during the above experiments, there was still reason to expect a significant degree of such effects if similar doses are given without the protection of barbiturate anesthesia. Accordingly, a series of intravenous injections were given in eight normal, unanesthetized dogs, while ECG records were taken before and after each injection. In half these trials, the sequence of injections was 1, 10, 50, and 50 mgm./kgm., administered over periods varying from one to two and a half hours. This amounted to a total of 111 mgm./kgm., administered in periods of two and a half hours or less. In the other half of the experiments, only the first two or three injections of this series were made. Doses of 1 mgm./kgm. usually showed no effects or mild manifestations of the same effects as with larger doses. With the 10 and 50 mgm./kgm. doses, the characteristic initial effect was first a moderate bradycardia, often with ventricular or nodal rhythms. At other times, or subsequently, there would be tachycardia and showers of ventricular ectopic beats. Ventricular ectopic tachycardia usually disappeared in one hour or less. If ectopic tachycardia was produced by one dose, a subsequent dose of the same size might not produce as much irregularity as the first. All dogs survived these injections and two hours after the last injection usually showed approximately normal rhythm and rate. By comparison, ephedrine in doses of 30 mgm./kgm. under similar conditions usually produces a profound bradycardia.

*Effects on isolated heart preparations* In experiments with isolated heart preparations, it has been general policy to ignore the fact that changes of various sorts and particularly drug changes, can affect the integrity of the aortic valves and thereby affect conclusions based simply on measurement of total perfusion flow. (Changes of flow in the Thebesian vessels or channels such as those described by Weirn are probably negligible factors.) This disturbing factor of aortic valve leakage was demonstrated by Wiggers (9) in 1909 and has been more recently recognized by Winder and Karsel (10). We have for some time been experimenting with determinations of this sort, primarily for the purpose of obtaining a more definite demonstration of the coronary flow changes occurring during the phase of depressant action by sympathomimetic amines. It is logical to consider that coronary spasm may be a causative factor in this typical

depressant action. Previous experiments have shown that the over-all perfusion flow does not indicate any important degree of coronary spasm during the effect. Nevertheless, it was considered desirable to demonstrate that changes in aortic valve leakage did not vitiate the correctness of such conclusions. Our observations have not conflicted with the prevailing opinion that depression by sympathomimetic amines is due primarily to some other factor than coronary spasm. We have, in fact, found that coronary flow frequently increases during the periods of depressed stroke amplitude. Further, we find that it is only infrequently that aortic valve leakage develops to such degree as to vitiate conclusions regarding coronary flow changes. The effect, however, does occur, and it would probably be well to make this additional determination in those instances where determinations of coronary flow are of critical value.

The effects of Aranthal were compared along with those of Octin and ephedrine. It is generally true that each of these three amines will cause an increase of amplitude stroke and rate most readily on their first application. Subsequent

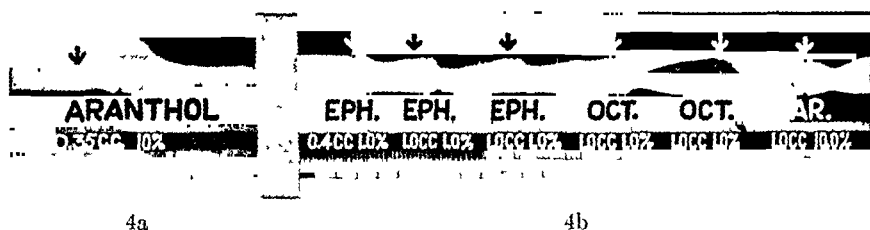


FIG. 4a. Isolated rabbit heart preparation. (1) Heart lever tracing. (2) Dropwise record of ordinary perfusion outflow. (Changes thus recorded can not be recognized when tracing is reduced for publication.) (3) Dropwise record of flow from accumulation in left ventricle (4) Time in minutes. Aranthal HCl was injected into perfusion tube near the heart in dosage of 0.35 cc. 10 per cent solution during interval of about 40 seconds. Perfusion flow rate about 10 cc./min.

FIG. 4b Same recording and conditions as fig. 4a. Comparison of effects of ephedrine, Octin, and Aranthal hydrochlorides. (Ephedrine HCl 1 per cent; Octin HCl 1 per cent; Aranthal HCl 10 per cent.)

applications will cause stimulation of lesser degree or outright depression. Still further applications of each of these causes relatively uniform degrees of depression and can be repeated a considerable number of times with only moderate acceleration of the natural decline in activity of this heart preparation. This has made it possible to repeat comparisons of these three drugs several times in the course of one experiment. When injected into the perfusion fluid at a point near the aortic cannula, Aranthal on first application usually increases stroke amplitude and rate. With perfusion flow rates of approximately 10 cc./min. the effect can ordinarily be obtained with amounts of 0.2 to 0.5 cc. of 1 per cent to 10 per cent of the hydrochloride (see fig. 4a). Subsequent injections of these or larger amounts will produce a lesser degree of stimulation or clear depression. When compared on the basis of a 1 per cent solution of Octin hydrochloride, a 1 per cent solution of ephedrine hydrochloride, and a 10 per cent solution of Aranthal hydrochloride, the Aranthal causes a more frequent and greater degree of stimulation on first application and a lesser degree of depression on subsequent

applications. The difference is distinct but moderate (see figs. 4b and 5). Under these conditions, Octin is somewhat more depressant than ephedrine. The relative molecular concentrations of base used here are: Ephedrine 1.0, Octin 1.15, and Aranthal 10.4. Coronary flow is increased to a somewhat greater degree with Aranthal than with the others, although the distinction is not great. In the case of each of the three amines, coronary flow may be increased during the interval of depression and, accordingly, this depressant action must be interpreted on the basis of some other effect than coronary spasm.

The relations between cardio-vascular effects of these three amines, thus observed with the isolated heart preparation, are in agreement with the relations observed in the open-chest experiments with dogs.

*Local vasoconstrictor action.* Aranthal did not appear to be a very marked vasoconstrictor as judged by our circulatory experiments in dogs and by determination of coronary perfusion rates in isolated rabbit hearts. This impression

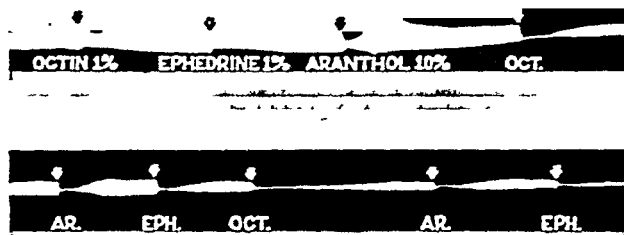


FIG. 5 Same recording and conditions as before. Drugs given throughout in 1.0 cc. volume of solution

has been borne out by more direct observations using a set of conditions which simulate one of the more common applications of local vasoconstrictors, that is, the use as a "chemical tourniquet" when injected along with large subcutaneous doses of cocaine. A series of ten dogs were injected with cocaine hydrochloride in doses of 30 mgm., 1 gm. subcutaneously. With equal volumes of solution, varying concentrations of Aranthal were also included in the injected solutions. The concentrations required to give effective protection against the typical systemic effects were in the range of 10 per cent and 20 per cent. Lower concentrations permitted the development of typically extreme excitation effects, which were controlled with pentobarbital. Under these same conditions, epinephrine is effective in concentrations of 1:25,000 to 1:10,000.

*Clinical trials.* A total of about twenty intravenous injections with Aranthal were made in seven patients by Dr. J. A. Boone and Dr. F. M. Ball. Doses up to 3 cc. of the 10 per cent solution injected over periods of about two minutes

produced no clear effects although one patient, an instance of Class IV decompensation, exhibited brief showers of premature contractions. There were some suggestive indications of improvement in conditions of heart failure. This series can be taken, however, to indicate no more than that this drug administered on a weight basis exhibited less marked hypertensive effects in patients than in animals under the described experimental conditions. Further clinical trials of a more extended nature are being conducted by Dr. George J. Thomas.

DISCUSSION. Sympathomimetic compounds have been generally recognized as producing intense stimulation of cardiac contractile force. The effects, however, have ordinarily been considered too brief and too much associated with pressor effects to warrant consideration for use in conditions of heart failure due to disease. Modification of these drugs in the direction of that which has been shown for Aranthal would indicate better possibilities for such application in the more acute types of heart failure. A wider selection of drugs in this category, with demonstrated cardio-vascular characteristics, should prove advantageous in the management of anesthetic and surgical emergencies as well as for other conditions in which synthetic variants of ephedrine are used. Among the latter may be mentioned their use in combatting the hypotension of spinal anesthesia (11-16), in increasing excitability of the heart in special instances of block (17, 18) and in the management of orthostatic hypotension (19). Another suggested application of this sort is in the type of circulatory failure due to reflex arteriolar dilation such as has been described as occurring after penetrating stab wounds of the chest (20).

#### SUMMARY

2-Methylamino-6-hydroxy-6-methyl heptane produced in dogs pressor effects and cardiac stimulation when given in doses five to ten times greater than those producing comparable effects with ephedrine. In contrast to ephedrine and 6-methylamino-2-methyl-2-heptene, this compound produced a distinctly lesser degree of cardiac depression when given in large or repeated doses. Similar relations of cardiac depression for these three amines were demonstrated in the isolated rabbit heart, although the distinctions were not so pronounced.

#### REFERENCES

1. JACKSON, D. E.: *Anesth. & Analg.*, **26**: 1, 1947.
2. JACKSON, D. E.: *Anesth. & Analg.*, **27**: 61, 1948.
3. AHLQUIST, R. P.: *THIS JOURNAL*, **85**: 283, 1945.
4. WALTON, R. P., PREACHER, C. B., AND CANNON, W. M.: *THIS JOURNAL*, **92**: 214, 1948.
5. WALTON, R. P., AND BRODIE, O. J.: *THIS JOURNAL*, **90**: 26, 1947.
6. WALTON, R. P., BELKIN, M., AND BRODIE, O. J.: *Fed. Proc.*, **6**: 380, 1947.
7. HUGGINS, R. A., HANDLEY, C. A., AND LAForge, M.: *Proc. Soc. Exp. Biol. & Med.*, **68**: 543, 1948.
8. BRILL, H. H., COTTEN, M. DE V., AND WALTON, R. P.: *Fed. Proc.*, April, 1949.
9. WIGGERS, C. J.: *Am. J. Physiol.*, **24**: 391, 1909.
10. WINDER, C. V., AND KAISER, M. E.: *THIS JOURNAL*, **93**: 86, 1948.
11. LORHAN, P. H., AND MOSSER, D.: *Ann. Surg.*, **125**: 171, 1947.

- 12 DRIPPS, R. D., AND DEAMING, M. VANN. *Surg Gynec & Obst*, **83**: 312, 1946
- 13 LA COMPTE, C. B. *Anesth & Analg*, **25**: 168, 1946
- 14 ROMAN VIGA, D. A., AND ADRIANI, J. *Anesthesiol*, **7**: 62, 1946
- 15 DODD, H., AND PRISCOTT, F. *Surg Gynec & Obst*, **77**: 645, 1943
- 16 GOLD, H. *Cornell Conference on Therapy*, Vol. 2, 146, 1947
- 17 NATHANSON, M. H., LANGELBERG, H., AND HERSH, J. *Am Heart J*, **24**: 153, 1942
- 18 GREENE, J. A., AND BENNETT, A. W. *Am Heart J*, **30**: 415, 1945
- 19 KORSS, H. M., AND RANDALL, W. L. *Ann Int Med*, **12**: 253, 1938
- 20 MERRILL, A. J., WARREN, J. V., STAD, E. A., JR., AND BRANSON, L. L. *Am Heart J*, **31**: 413, 1946



# FURTHER PHARMACOLOGIC CHARACTERIZATION OF THE SYMPATHOMIMETIC, ALIPHATIC AMINE 2 METHYLAMINO-6-HYDROXY-6-METHYL HEPTANE<sup>1, 2</sup>

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There have been a number of recent reports making reference to the general status of the pharmacology of aliphatic amines (1-8) and aralkyl amines (9). One of these, 2 methylamino-6-hydroxy-6-methyl heptane (EA-83) (Aranthol), has been particularly described with respect to its cardio-vascular action (10). The present study was carried out as an extension of the pharmacologic characterization of this particular amine.

**EXPERIMENTAL.** The drug was used in the form of its hydrochloride or mucate salts, both of which are readily soluble in water. Unless otherwise indicated, doses reported here refer to those of the hydrochloride salt. All doses are expressed in terms of mgm./kgm. body weight. In the case of table 1, doses are expressed on the basis of the free base, which represents 81 per cent of the hydrochloride salt and 60 per cent of the mucate salt.

**RESULTS. *Acute Toxicity.*** Determinations of acute toxicity are given in table 1. Estimated figures for LD<sub>50</sub> and the corresponding upper and lower limits of error were calculated according to the procedure described by DeBeer (11). These computations were made by Mr. M. U. Dantzler.

In dogs receiving the smallest doses (50, 70 mgm./kgm. intravenously) there was some limited development of manifestations which resembled those of Octin previously described (6) (wagging tail, licking lips, shaking head). These latter effects were not noted in the dogs receiving the larger doses which characteristically produced prompt development of hyperpnea, pilomotor reactions (raising of hair over the back of the neck), emesis, defecation, micturition and occasional salivation. With doses of 200 to 280 mgm./kgm. the effects were more extreme in character and involved marked tremors, tonic seizures, arching of the neck, and marked hyperpnea. In this and the higher dosage range, death was characterized by respiratory arrest with later cessation of the heart beat. Artificial respiration would maintain the heart beat. On post-mortem examination the lungs and liver were markedly congested. Examination of those dogs dying one or more days after the injection consistently showed pneumonic consolidation.

It was concluded from these observations with dogs that the immediate deaths were due to excessive central nervous system stimulation followed by depression.

<sup>1</sup> Reported in abstract in Fed. Proc., 6: 380, 1947.

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<sup>3</sup> Now at the National Cancer Institute, Bethesda, Maryland.

TABLE 1

DOSE CALCULATED AS FREE BASE	NUMBER ANIMALS	PER CENT SURVIVAL	ESTIMATED LD <sub>50</sub>	CALCULATED LIMITS OF ERROR	SURVIVAL TIME
Intravenous injection of the mucate in Swiss mice (temp. 30-32°C.)					
<i>mgm /kgm</i>					
120	6	100			
180	11	82			
195	17	41			
210	4	0			
210	5	0	192	185-199	
Intravenous injection of the mucate in rabbits (temp. 26-30°C.)					
120	4	100			
180	8	87			
210	8	50			
210	12	0	210	191-231	
Intravenous injection of mucate and hydrochloride in dogs					
50, 70, 150, 180, 190	5	100			
200	6	66			12 hrs.; 48 hrs.
215	1	0			60 hrs.
250	2	0			3 hrs.; 24 hrs.
280	1	0			½ hr.
380	2	0			3 min.; 2 hrs.
400	2	0			4 min.; 4 min.
500	2	0	210	—	3 min.; 4 min.
Subcutaneous injection of the mucate in Swiss mice (temp. 25-26°C.)					
510	6	83			
900	12	75			
1200	16	70			
1500	16	50			
1950	16	19	1320	1051-1653	
Oral administration of the mucate to Swiss mice (temp. 26-28°C.)					
900	6	100			
2400	14	93			
3000	14	61			
3600	6	16	3091	2844-3357	

The late deaths were evidently due to pneumonia, probably as a sequel to lung edema.

*Effects on central nervous system in lower doses.* In a series of seven dogs,

anesthesia approximately at the level of Stage III, Plane i (Guedel) was produced by intravenous administration of sodium pentobarbital in doses of 25 mgm./kgm. After periods of 40 to 80 minutes, the condition of the dogs was noted with regard to activity of corneal reflexes, tendon reflexes, and respiratory rate. The drug was then injected intravenously in doses of either 10 mgm./kgm. or 20 mgm./kgm. of the hydrochloride. The typical response was a slight depression of reflexes lasting for 30 to 60 minutes, some slight increase in respiratory rate and an approximately normal recovery time; commonly, the heart appeared to be beating more forcefully as judged by the apex thrust and rhythmic vibration of the head and limbs. In concluding that the drug in this dose had no recognizable analeptic effect, it may be pointed out that ephedrine under the conditions just described, exhibits a fairly consistent and marked analeptic effect.

*Mydriasis by local application.* Experiments in six rabbits demonstrated that 10 per cent and 20 per cent solutions of the mucate produced a limited degree of mydriasis. In all cases, the changes in pupillary size were less than those produced by solutions of ephedrine hydrochloride of either 2 per cent or 10 per cent concentration applied at the same time in the contralateral eye. Both 10 per cent and 20 per cent concentrations of the mucate produced recognizable local irritation.

*Effects on the gut.* No significant influence was observed on the tracing patterns obtained by the use of balloons arranged to record changes of intra-luminal pressure in gut fistulae segments. In each of six dogs prepared with such Thierry-Vella fistulae, no significant effects were observed following the intravenous injections of doses of the hydrochloride ranging from 1 to 10 mgm./kgm. In this respect, the effects of Aranthal resemble those of ephedrine.

*Local effects at site of injection.* Following subcutaneous injections, no local effects were noted with concentrations up to 5 per cent. With solutions of 10 per cent to 20 per cent of the mucate, occasionally there was skin induration, which in turn, would, in some cases, proceed to ulceration. This occurred in the mice which received relatively large volumes by the subcutaneous route. A few experiments in rabbits indicated that the same type of effects could be obtained on occasion with concentrations of 10 per cent or greater if the doses were greater than 25 mgm./kgm. of either the hydrochloride or mucate. At the same time, there might also be complete absence of such effects with doses of 100 mgm./kgm.

*Chronic toxicity.* Two groups of Swiss mice, one made up of eighteen mice and the other of twelve mice, were maintained for 90 days. The drinking water of the first group contained 0.5 per cent of the mucate while the second group did not receive any of the drug. In the case of the first group, the average calculated daily intake was 800 mgm./kgm. of the free base. No significant differences were noted in the two groups on the basis of weight curves, reproductivity, or gross and microscopic autopsy. About one-hundred representative tissue sections were examined.

Six puppies (weight 2.3 to 3.8 kgm.) were given 120 mgm./kgm. daily of the

free base (as the mucate, in two divided daily doses, orally in capsules). One died in two weeks and two in four weeks, with distinct pneumonic consolidation in each case. The other three were maintained on this dose for 90 days, when they were sacrificed. They gained weight satisfactorily and appeared to be in good condition at the end of the period, except for a limited degree of patchy pneumonic consolidation in one case. Tissue sections in all dogs showed nothing significant beyond the pneumonic processes mentioned. It is problematic whether or not the drug influenced the incidence of pneumonia in these cases, since puppies commonly have a high incidence of such infections.

#### SUMMARY

2 Methylamino-6-hydroxy-6-methyl heptane was found to be of a low order of chronic toxicity and to be without important species differences with regard to acute toxicity. The  $LD_{50}$  of intravenously injected doses in mice, rabbits and dogs was approximately 350 times greater than the dose which has been described as producing in dogs an approximately 30 per cent increase in contractile force of the heart (10). Acute toxicity appeared to be based chiefly on central nervous system stimulation, although in animals surviving for some hours after large intravenously administered doses, lung consolidation was commonly observed. The drug exhibited weak mydriatic action, relative absence of effect on gut movements recorded with intestinal fistula dogs and relative absence of effects on central nervous system when used in the lower dose range.

#### REFERENCES

1. SWANSON, E. E., AND CHEN, K. K.: *THIS JOURNAL*, 88: 10, 1946.
2. SWANSON, E. E., AND CHEN, K. K.: *THIS JOURNAL*, 93: 423, 1948.
3. CHEN, K. K.: *Ann. Otol. Rhinol. & Laryngol.*, 57: 287, 1948.
4. JACKSON, D. E.: *Anesth. & Analg.*, 26: 1, 1947.
5. JACKSON, D. E.: *Anesth. & Analg.*, 27: 61, 1948.
6. WALTON, R. P., PREACHER, C. B., AND CANNON, W. M.: *THIS JOURNAL*, 92: 214, 1948.
7. BEYER, K. H.: *Physiol. Rev.*, 26: 169, 1946.
8. MARSH, D. F.: *THIS JOURNAL*, 94: 225, 1948.
9. WINDER, C. V., ANDERSON, M. M., AND PARKE, H. C.: *THIS JOURNAL*, 93: 63, 1948.
10. WALTON, R. P., AND BRODIE, O. J.: *THIS JOURNAL*, 96: 343, 1949.
11. DEBEER, E. J.: *THIS JOURNAL*, 85: 1, 1945.

# DETERMINATION OF HOMOSULFANILAMIDE HYDROCHLORIDE (SULFAMYLON HYDROCHLORIDE) WITH OBSERVATIONS ON ITS ABSORPTION IN DOGS AND MAN<sup>1</sup>

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p-Aminomethylbenzenesulfonamide hydrochloride (also known as homosulfanilamide hydrochloride, Sulfamylon<sup>2</sup> hydrochloride, Marfanil, Mesudin, ambamide) was apparently first synthesized in Germany about ten years ago (1). It was also prepared in this country by Miller *et al.* (1a), but since it was found not to be significantly active against streptococcal infections in mice, its potentialities were not investigated further by this group. Later its effectiveness against a variety of anaerobes, and some Gram-negative organisms which are otherwise sulfonamide-resistant, came to be recognized (2-5). It has been used chiefly for the treatment of infected wounds or, in general, those situations where local therapy is indicated (6-11). During the war the drug was used extensively by the Germans and when this fact became generally known (12), a new impetus to its study was supplied. It has, however, continued to be used chiefly for local application rather than for systemic therapy, probably because it undergoes rather rapid deamination in the animal body with consequent loss of activity (13-16). The fact that it is not antagonized by p-aminobenzoic acid is well known (17). Interest in the drug has been stimulated recently by the fact that it has given excellent results in the treatment of certain types of infections, particularly when used in conjunction with streptomycin (18), or with other sulfonamides (7, 19-25). The determination of Sulfamylon hydrochloride in biological materials has always been a difficult problem because its amino group is aliphatic in character and the familiar Bratton-Marshall method (26) is not applicable. In 1945 Siebenmann and Plummer (27) proposed a method for determining the drug in biological fluids. This method was based on the ability of Sulfamylon hydrochloride to inhibit the growth of *Clostridium histolyticum* on blood agar plates. The limitations of this type of analysis are obvious but it did enable Siebenmann and Plummer to ascertain that: 1) the drug is absorbed rapidly when administered orally or intramuscularly, and 2) only a small portion of the dose so administered is excreted in the urine in an active form.

More recently Heideman and Rutledge (28) have published a spectrophotometric method applicable to biological fluids, based on the absorption of Sulfamylon at 265 m $\mu$ . Percentage recoveries of the drug when added to various biological materials were: spinal fluid, 81; ascitic fluid, 80; blood serum, 78; whole

<sup>1</sup> Presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics at Detroit, April 22, 1949.

<sup>2</sup> Registered trademark of Winthrop-Stearns Inc., brand of p-aminomethylbenzenesulfonamide.

blood, 61. This method is complicated by some technical difficulties, the principal one being that the two precipitating agents,  $\text{ZnSO}_4$  and  $\text{Ba(OH)}_2$ , must be perfectly balanced if high results are to be avoided. The large correction factor which must be applied to whole blood is clearly objectionable in principle. A more serious disadvantage is the fact that the deamination product, p-carboxybenzenesulfonamide, absorbs light in the same region of the spectrum, and absorbs more strongly than the parent drug, as is shown in fig. 1. This means that a blood sample which contained, for example, 15 mgm. per cent each of Sulfamylon

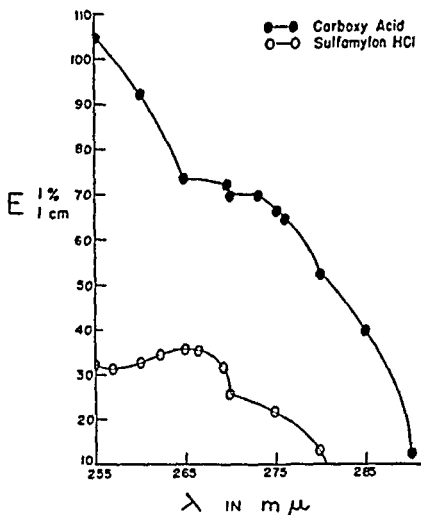


FIG. 1. Partial ultraviolet absorption spectra of Sulfamylon hydrochloride and its deamination product, p-carboxybenzenesulfonamide. The data were kindly supplied to us by Dr. F. C. Nachod. The carboxy acid was isolated and crystallized from the urine of dogs which had received large doses of Sulfamylon hydrochloride orally. M. p. 286–288°C. (corr.). In literature (16), 296°C.

hydrochloride and its metabolite would be found to contain about 50 mgm. per cent by the Heideman-Rutledge method. It seems likely that a large portion of what they were measuring, particularly in the latter stages of absorption, was actually the metabolite. Rutledge and Heideman (29) gave values for the blood levels of human subjects receiving dosages of 400–600 mgm./kgm. orally or subcutaneously. Peak values of 30–40 mgm. per cent were indicated. They concluded that comparatively high dosage at rather frequent intervals is needed to maintain blood levels at or above 10 mgm. per cent. There were many un-

pleasant side reactions following oral administration of the drug, but there was no evidence of damage to the kidneys or other organs.

Since the amino group of Sulfamylon hydrochloride is aliphatic and primary, it gives the Ehrlich-Herter reaction (30), which is the basis for the Folin colorimetric method for amino acids (31). It was observed by one of us (M.E.A.) that it is possible to differentiate Sulfamylon hydrochloride from amino acids, however, on the basis of the following property: if instead of acidifying after the completion of the condensation reaction (as is done in the Folin method or its more recent modifications) (32, 33) the reaction is kept definitely alkaline (pH 9-10), the colored compound produced by the interaction of Sulfamylon hydrochloride and  $\beta$ -naphthoquinone sulfonate may be extracted rather specifically by certain organic solvents. Of some 30 different solvents tested, methyl *n*-amyl ketone (Eastman Kodak Co. No. 2185) proved to be the most specific. Under the stated conditions alanine, for example, gives no extractable color whatsoever, and blood filtrates have only a rather small blank value. Thus far no attempt has been made to apply the method to such materials as spinal or ascitic fluid. It has not been successful when applied to urine since the blank value is entirely too high to be satisfactory<sup>3</sup>. Details of the procedure for whole blood follow:

1. To 25 cc. water add 3 cc. blood, allow time for laking of the red cells, then add 2 cc. of 25 per cent metaphosphoric acid (Merck or Mallinckrodt, analytical reagent grade. This reagent should be prepared daily, or weekly if kept in the refrigerator). Shake well, and let stand for about 30 min.

2. Centrifugate, then filter through Whatman No. 1 filter paper.

3. To 20 cc. of filtrate in a 50 cc. g.s. graduated cylinder add enough approx. 10 per cent NaOH to neutralize to phenolphthalein. This quantity should be determined on a separate portion of the filtrate (for example, by titrating 2 cc. of the filtrate with a 1:10 dilution of the 10 per cent NaOH). It need be determined only once since it will apply to all filtrates: Add 2 cc. of an aqueous buffer solution (containing 4 per cent each of  $\text{NaHCO}_3$  and  $\text{Na}_2\text{CO}_3$ ), followed by 1 cc. of freshly prepared aqueous 1 per cent sodium  $\beta$ -naphthoquinone sulfonate. Stopper, mix, and let stand at room temperature for 30 min.

4. Add 2 cc. of a 5 per cent solution of sodium ascorbate or isoascorbate. (This may be prepared by weighing out 1 gm. ascorbic acid and 500 mgm.  $\text{NaHCO}_3$  and gradually adding water to make a volume of 20 cc. This solution is prepared shortly before use.) Mix and allow to react about 2 min.

5. Add exactly 10 cc. methyl *n*-amyl ketone. Shake mechanically for 5 minutes, then separate the organic layer; if an emulsion forms, break it by centrifugation.

6. Dry the ketone extract over about 100 mgm. anhydrous  $\text{Na}_2\text{SO}_4$ . Read the color in a photoelectric colorimeter using a 420 m $\mu$ . filter.

*Calibration of colorimeter.* Into a series of graduated cylinders measure various amounts of aqueous Sulfamylon hydrochloride solution to cover the range 0-1.25 mgm. in eight or ten equal steps. Add to each 1 cc. of 25 per cent  $\text{HPO}_3$ , plus enough 10 per cent NaOH to neutralize this amount of  $\text{HPO}_3$  to phenolphthalein). Add water to the 20 cc. mark and 2 cc. of the buffer solution. Now add 1 cc. of the naphthoquinone sulfonate and proceed according to Steps 4-6 above. The colors are read as described, using fresh solvent for a blank setting. The relationship of optical density to concentration is in accordance with Beer's law.

<sup>3</sup> The blank may be decreased to the equivalent of about 5 mgm. per cent by aerating off the ammonia and adsorbing interfering substances on Decalco. However, it is difficult to standardize the conditions so that Sulfamylon hydrochloride is not also adsorbed.

*Notes on procedure.* As described, the method is adaptable to the Evelyn colorimeter, using the large colorimeter tubes which require 9-10 cc. of solution. If a different type of instrument is available, in which 5 cc. of solution or less can be read, the amount of blood filtrate may be reduced from 20 cc. to 10 cc., and all of the subsequently prescribed quantities are cut in half.

If the amount of Sulfamylon hydrochloride in the sample is expected to be very large, it is better to use only half (or even one-fourth) of the amount of filtrate directed, and the corresponding amount of 10 per cent NaOH. Water is then added to the 20 cc. mark, and all other quantities of reagents are kept the same. This must be borne in mind in making the calculations: from each observed optical density is subtracted the entire reagent blank, but only one-half (or one-fourth) of the difference between the reagent blank and a blank, or control determination, on the blood of the animal. Usually the optical density of the

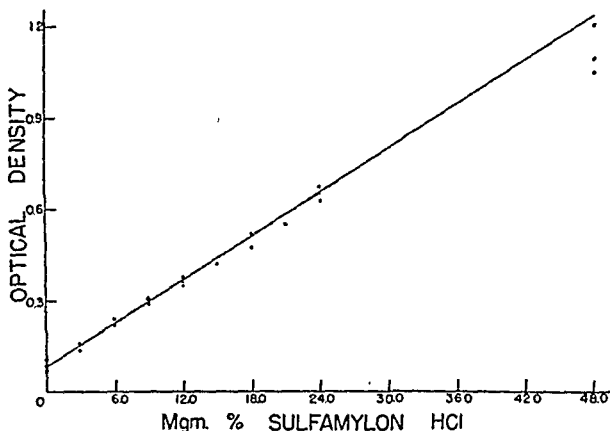


FIG. 2. Recovery of Sulfamylon hydrochloride added to whole blood in a number of different concentrations. The plotted points represent the observed optical densities for the individual samples, and the curve represents the position calculated for 100 per cent recovery.

reagent blank is 0.03-0.04, and that of a blood blank is 0.08-0.11. It is appropriate to mention at this point that the purpose of adding sodium ascorbate is to eliminate a large reagent blank which would otherwise be obtained as a result of a reaction between metaphosphoric acid and naphthoquinone sulfonate (34). Probably other reducing agents would serve the purpose, but no others were tried after this one was found to be successful.

Recoveries of Sulfamylon hydrochloride added to blood are excellent by this procedure. Over the range 0-30 mgm. per cent the recoveries average  $99 \pm 5$  per cent. (As pointed out above, the blank value of dog or beef blood is quite consistent; it corresponds to 2-3 mgm. per cent Sulfamylon hydrochloride of which 30-40 per cent is due to the reagents.) If the concentration is higher the recoveries begin to fall off, but if smaller amounts of the blood filtrate are used, the results are just as satisfactory. Typical recoveries from blood are plotted against the theoretical curve in fig. 2.

**STUDIES ON ABSORPTION.** Studies of the blood levels of Sulfamylon hydrochloride, following oral or parenteral administration to dogs, were now made. As



far as possible, the same dogs were used throughout in order that comparisons could be made for at least two different routes of administration on the same animal. We wished first of all to see how high the blood levels would be following a rapid intravenous infusion, and how long the drug would be retained. The solution used for the infusion contained 10.0 gm. Sulfamylon hydrochloride and 0.35 gm. Sulfamylon in water to make 275 cc. of solution. Such a solution has a pH of 7.35 and is almost exactly isotonic with blood. This solution was given in the amount of 15.8 cc./kgm., and is the equivalent of 600 mgm./kgm. of Sulfamylon hydrochloride. The dogs used were under pentobarbital anesthesia of sufficient depth to keep them quiet until the infusion was completed.<sup>4</sup> The results are presented graphically in fig. 3.

When the infusions were completed in 30 minutes they resulted in blood levels of 80–100 mgm. per cent Sulfamylon hydrochloride at the end of the infusion. From four to six hours were required to remove the drug completely from the blood. An infusion which covered a period of four hours gave a much lower peak level, and clearance was complete in about 90 min. The rate of clearance in all these cases seems very rapid when compared to that observed following the oral administration of sulfanilamide (35, 36) or following the intravenous administration of sulfanilamide or sodium sulfapyridine (37). The same graph includes the results of one subcutaneous infusion. The infusion was completed in 90 min., and a peak blood level of 9 mgm. per cent resulted, but there was only a slight decrease in concentration up to five hours later. This dog was under both pentobarbital and barbital anesthesia, as was the animal which received the four-hour intravenous infusion.

Equivalent oral dosages were given to some of the same animals (by stomach tube, in milk), with the results shown in fig. 4. Peak blood levels (9–10 mgm. per cent) were attained in about two to three hours after medication, and the absorption appeared to be quite rapid.

The effect of repeated oral doses was also studied, with the results shown in fig. 5. The dogs received doses of 150 mgm./kgm. every four hours until twelve doses had been given. These medications were given as crystals in gelatin capsules. The blood levels fluctuated, but were usually of the order of 4–5 mgm. per cent. Clearance of the blood after the last dose was rapid.

<sup>4</sup> The pharmacodynamic effects of intravenous infusions have been studied in our laboratory by Dr. F. P. Ludueña and Miss E. Ananenko and have been described as follows:

The intravenous infusion of a total dose of 600 mgm./kgm. of Sulfamylon hydrochloride (3.8 per cent sol.) in two barbitalized dogs (within 22–25 minutes) produced a rapid fall in blood pressure of 60–90 mm.Hg within the first two to five minutes of infusion. From this low level the pressure started rising although the drug infusion continued.

This hypotensive effect of Sulfamylon hydrochloride was apparently of vascular origin as the heart rate was only slightly decreased during the drug administration. An increase in respiratory rate was observed, probably due to the fall of pressure. There was also a decrease of the tonus of the small intestine.

In another anesthetized dog the same dose of Sulfamylon hydrochloride was administered intravenously at a much slower rate (total dose administered in about four hours). Again the blood pressure fell, starting 30 minutes after the beginning of the infusion, but the maximum fall was only 35–40 mm. Hg.

Other experiments, in which dogs received intravenous or single oral doses of 600 mgm./kgm. of Sulfamylon hydrochloride, were conducted before the blood method had been entirely perfected. They gave substantially the same results

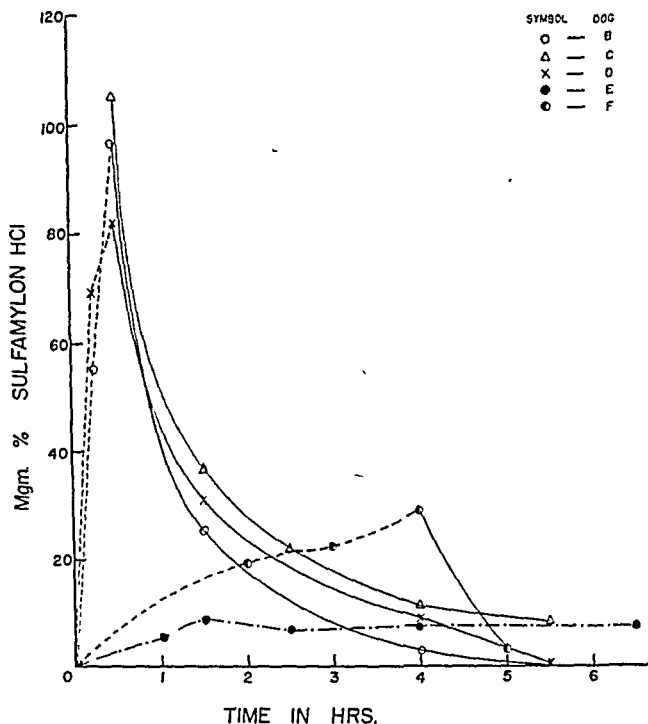


FIG. 3. Whole blood concentrations of Sulfamylon hydrochloride resulting from the infusion of an isotonic solution (3.8 per cent), pH 7.4. Total dose was 600 mgm./kgm. of the drug. The dotted line represents observations made during the infusion; the solid line represents observations made after the completion of the infusion. The dot-dash line shows blood concentrations in a dog which received the same medication by subcutaneous infusion starting at 0. The total time required for the infusion was 90 minutes.

as those which are reported in this paper, but the data are not included because they are not as accurate.

Several human subjects were given oral doses of about 110 mgm./kgm.

Sulfamylon hydrochloride orally in milk: the distastefulness of the product and its side reactions mitigated against the use of any higher dose level although

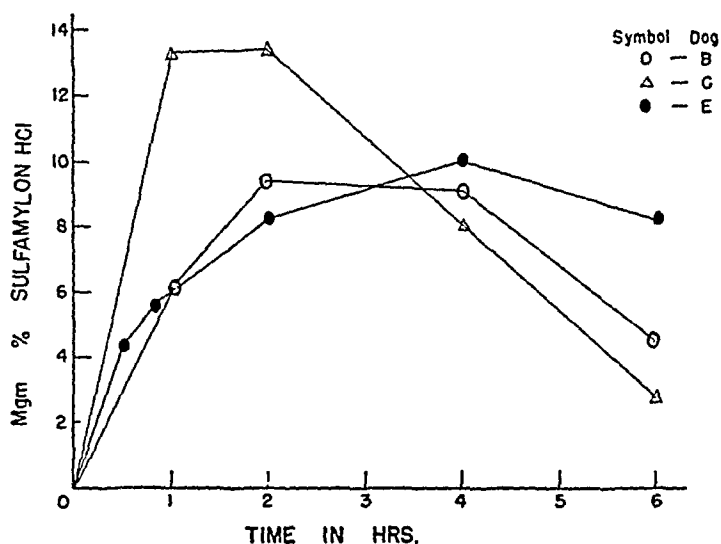


FIG. 4. Whole blood concentrations of Sulfamylon hydrochloride following single oral doses of 600 mgm./kgm.

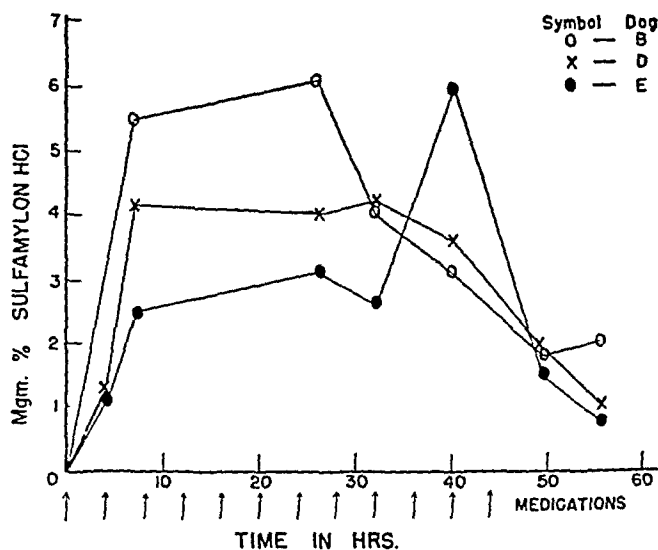


FIG. 5. Whole blood concentrations of Sulfamylon hydrochloride resulting from the administration of twelve oral doses of 150 mgm./kgm., given at intervals of four hours.

as much as five to six times this amount has been given (28). Blood concentrations were determined in these subjects over a period of four hours after medication; the highest level achieved was only 2-4 mgm. per cent.

**DISCUSSION.** It is evidently difficult to achieve high blood levels of Sulfamylon hydrochloride by any route other than the intravenous. By single oral doses of 600 mgm./kgm. the highest levels observed were only 10–12 mgm. per cent, and it would appear that it would require at least 300 mgm./kgm. (if not more) every four hours to maintain such a level. Although only one subcutaneous infusion was carried out, it does not appear to offer the possibility of achieving any higher blood level than 10–12 mgm. per cent. These results are in marked contrast with those of Marshall, Emerson and Cutting (35) who obtained blood levels of 10–12 mgm. per cent sulfanilamide by the oral administration of 100 mgm./kgm. to dogs. Since only small amounts of Sulfamylon hydrochloride are found in the urine following oral administration (27) it seems likely that rapid deamination of the drug accounts for the fact that it shows a lesser tendency to accumulate in the blood. Even making allowance for the fact that Heideman and Rutledge were working with human subjects rather than dogs, it seems strongly indicated that the high blood levels they observed were due in large part to the presence of p-carboxy-benzenesulfonamide.

#### SUMMARY

A colorimetric method for the determination of homosulfanilamide hydrochloride (Sulfamylon hydrochloride) in blood is presented. The compound is allowed to react with sodium  $\beta$ -naphthoquinone sulfonate in alkaline solution, and the yellow condensation product is extracted by means of methyl n-amyl ketone. The optical density of the extracts, measured at 420 m $\mu$ , is proportional to the amount of Sulfamylon hydrochloride present. The method has been applied to the study of blood levels in dogs and men following oral or parenteral administration. Oral doses of 600 mgm./kgm. to dogs give peak blood levels of about 10 mgm. per cent at from two to three hours after administration. Absorption and excretion and/or metabolism are rapid, and it would be necessary to give doses about every four or five hours in order to maintain this level.

Doses of 150 mgm./kgm. given every four hours serve to hold the blood level at about 4 mgm. per cent with occasional values as high as 6 mgm. per cent. By intravenous infusion of 600 mgm./kgm. in an isotonic solution (3.8 per cent, pH 7.4), within 30 minutes, blood levels as high as 100 mgm. per cent may be achieved without causing death although there is a marked fall in blood pressure; it requires five to six hours to reduce the concentration again to 0–3 mgm. per cent. Subcutaneous infusion of 600 mgm./kgm. in a solution of the same composition gives a peak blood level about the same as following an equal oral dose, but the return to zero concentration is much less rapid.

#### REFERENCES

1. KLARER, J.: German patent no. 726,386, appl. for Jan. 27, 1939; Chem. Zentr., 114: 978, 1943.
- 1a. MILLER, E., SPRAGUE, J. M., KISSINGER, L. W., AND MCBURNEY, L. F.: J. Am. Chem. Soc., 62: 2009, 1940.
2. KLARER, J.: Klin. Wochschr., 20: 1250, 1941; Domagk, G.: Klin. Wochschr., 21: 448, 1942.

3. SCHREUS, H. T., AND PELTZER, E.: *Klin. Wochschr.*, **20**: 504, 529, 1941; SCHREUS, H. T.: *Klin. Wochschr.*, **21**: 14, 671, 1942.
4. HAMRE, D. M., WALKER, H. A., DUNHAM, H. B., VAN DYKE, H. B., AND RAKE, G.: *Proc. Soc. Exper. Biol. and Med.*, **55**: 170, 1944.
5. LAWRENCE, C. A.: *J. Bact.*, **47**: 52, 1944.
6. BEYER, W.: *Zentralbl. f. Chir.*, **68**: 1730, 1941.
7. CLARK, W. B.: *Arch. Ophth.*, **38**: 683, 1947.
8. DOMAGE, G.: *Arch. klin. Chir.*, **205**: 120, 1943.
9. FELDT, A.: *Klin. Wochschr.*, **22**: 742, 1943.
10. BEYER, W.: *Münch. med. Wochschr.*, **88**: 1262, 1941.
11. Goecke, C. A.: *Münch. med. Wochschr.*, **89**: 542, 1942.
12. Editorial: *Lancet*, **1**: 635, 1944.
13. BEYER, K. H., AND GOVIER, W. M.: *Science*, **101**: 150, 1945.
14. BERNHEIM, F. H., AND BERNHEIM, M.: *Science*, **101**: 547, 1945.
15. HARTLES, R. L., AND WILLIAMS, R. T.: *Biochem. J.*, **40**: 24, 1946.
16. HARTLES, R. L., AND WILLIAMS, R. T.: *Biochem. J.*, **41**: 206, 1947.
17. JENSEN, K. A., AND SCHMITH, K.: *Z. Immunitäts.*, **102**: 261, 1942.
18. HOWES, E. L.: *Am. J. Med.*, **2**: 449, 1947.
19. HERRMANN, G.: *Münch. med. Wochschr.*, **90**: 697, 1943.
20. SCHUCHARD, W.: *Münch. med. Wochschr.*, **89**: 838, 1942.
21. CLARK, W. G., LEVEN, N. L., AND TSCUCHIYA, H. M.: *Comm. on Med. Res.*, PB40988: 4, 1944.
22. MITCHELL, G. A. G., REES, W. S., AND ROBINSON, C. N.: *Lancet*, **1**: 627, 1944.
23. SCHNEIDER, E.: *Deutsche med. Wochschr.*, **67**: 723, 1941.
24. STAFF, R.: *Deutsche med. Wochschr.*, **68**: 221, 1942.
25. WOJTA, H.: *Chirurg.*, **15**: 85, 1943.
26. BRATTON, A. C., AND MARSHALL, E. K., JR.: *J. Biol. Chem.*, **128**: 537, 1949.
27. SIEBENMANN, C. O., AND PLUMMER, H.: *THIS JOURNAL*, **84**: 291, 1945.
28. HEIDEMAN, M. L., JR., AND RUTLEDGE, R. C., JR.: *THIS JOURNAL*, **93**: 451, 1948.
29. RUTLEDGE, R. C., JR., AND HEIDEMAN, M. L., JR.: *J. Pediat.*, **33**: 274, 1948.
30. EHRLICH, P., AND HERTER, C. A.: *Z. physiol. Chem.*, **41**: 329, 1904.
31. FOLIN, O.: *J. Biol. Chem.*, **51**: 377, 393, 1922.
32. FRAME, E. G., RUSSELL, J. A., AND WILHELM, A. E.: *J. Biol. Chem.*, **149**: 255, 1943.
33. RUSSELL, J. A.: *J. Biol. Chem.*, **156**: 467, 1944.
34. REF. 32, p. 266.
35. MARSHALL, L. K., JR., EMERSON, K., JR., AND CUTTING, W. C.: *J. A. M. A.*, **108**: 953, 1937.
36. MARSHALL, E. K., JR., CUTTING, W. C., AND COVER, W. L.: *Bull. Johns Hopkins Hosp.*, **63**: 318, 1938.
37. MARSHALL, E. K., JR., AND LONG, P. H.: *J. A. M. A.*, **112**: 1671, 1939.

# THE ACTION OF SYMPATHOMIMETIC AMINES ON THE ISOLATED HEART OF THE FROG<sup>1</sup>

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The frog's heart isolated according to the technique of Straub appears to be a satisfactory tissue for studying the relationship of structure of 'sympathomimetic' agents to their action on a functioning muscular tissue essentially free of active adrenergic nerve fibers. This preparation responds regularly by inotropic changes, whereas chronotropic actions are less constant, due to the absence of or poor perfusion of the pace-maker tissues. Hoffman *et al.* (1) have shown that the isolated frog's heart is free of active chromaffin tissue. They found that the atropinized frog's heart does not respond to acetylcholine. The isolated atropinized hearts of mammals, however, are stimulated by acetylcholine and this stimulation is due to the release by acetylcholine of a sympathin-like substance.

This survey was undertaken in order to learn the requirements of the 'receptor substance' in terms of the chemical structures possessing sympathomimetic action on the heart. A fairly representative group of arylalkylamines and aliphatic amines has been tested.

Substances reputed to be adrenolytic agents have been examined for this activity on the isolated frog's heart. A satisfactory adrenolytic compound would allow the differentiation of drugs showing positive inotropic action into a group presumably acting on the same 'receptor' as epinephrine and another group with a more poorly defined action. However, in agreement with the presently accepted conclusion that the heart is resistant to adrenolytic action, none of the adrenolytic agents which were tested antagonized the action of epinephrine on this preparation.

**METHODS.** Frogs of the species *Rana pipiens* were used. Most of the experiments were performed between January and April. Hearts were prepared according to the technique of Straub, using the straight cannula of the type recommended by Fühner. The perfusion pressure was maintained at 5 cm. The modification of Ringer's solution employed by Krayer *et al.* (2) was used for the perfusion fluid and the dilution of drugs. Air was passed through the perfusion fluid. Room temperatures were between 21 and 26°C. In order to produce a slightly hypodynamic state, hearts were washed repeatedly for a minimum of two hours before the first tests were made.

In most cases dilutions of  $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$ , and  $1 \times 10^{-6}$  have been tested. In some instances this range has been extended to  $1 \times 10^{-7}$  and  $1 \times 10^{-8}$ . Each substance has been tested on from one to three hearts. In all but a few cases each compound has been tested on a separate heart. The order of testing the various dilutions was in the direction of increasing concentration. When there was no immediate response, contact was allowed for

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five minutes. Between tests the perfusion fluid was exchanged for fresh solution at least three times and a minimum of five minutes was allowed for recovery of the tissue.

In the course of this study the inotropic action of the following compounds has been tested (the numerical designations correspond to the compound numbers employed in the tables and body of the paper):

1. 2-phenylethylamine sulfate; 2. *l*-amphetamine sulfate; 3. desoxyephedrine hydrochloride; 4. *dl*-phenylalanine (neutralized with  $\text{NaHCO}_3$ ); 5. 1-(*p*-tolyl)-2-amino-propane sulfate; 6. tyramine hydrochloride; 7. hordenine sulfate; 8. 1-1-(4-hydroxyphenyl)-2-isopropylamino-ethanol tartrate; 9. paredrine hydrobromide; 10. 1-(4-hydroxyphenyl)-2-dimethylamino-propane hydrobromide; 11. 1-(4-hydroxyphenyl)-2-amino-propanol-1 hydrochloride; 12. *l*-tyrosine (neutralized with  $\text{NaHCO}_3$ ); 13. *l*-phenylephrine hydrochloride; 14. 1-1-(3-hydroxyphenyl)-2-methylamino-ethylamine hydrochloride; 15. epinephrine hydrochloride; 16. isopropyl norepinephrine (Isuprel); 17. kephrine hydrochloride; 18. 1-1-(3,4-dihydroxyphenyl)-2-methylamino-ethylamine hydrochloride; 19. 1-(3,4-dihydroxyphenyl)-2-amino-propane hydrobromide; 20. 1-(3,4-dihydroxyphenyl)-2-methylamino-propane hydrobromide; 21. 1-(3,4-dihydroxyphenyl)-alanine (neutralized with  $\text{NaHCO}_3$ ); 22. 1-(3,4-dimethoxyphenyl)-2-amino-propane hydrochloride; 23. 1-(3,4-methylenedioxyphenyl)-2-amino-propane hydrochloride; 24. 1-(*p*-anisyl)-2-amino-propane hydrochloride; 25. 1-cyclohexyl-2-amino-propane hydrochloride; 26. 1-cyclohexyl-2-methylamino-propane hydrochloride; 27. benzylamine hydrochloride; 28. 3-phenyl-propylamine-1 sulfate; 29. 4-phenyl-2-amino-butane sulfate; 30. 5-phenyl-2-amino-pentane sulfate; 31. 6-phenyl-2-amino-hexane hydrochloride; 32. 2-amino-ethanol (neutralized with hydrochloric acid); 33. isocamylamine hydrochloride; 34. 2-amino-4-methyl-hexane sulfate; 35. 2-amino-heptane sulfate (Tuamine); 36. 2-methylamino-heptane hydrochloride (Oenethyl); 37. 2-methylamino-6-methyl-heptene-2 hydrochloride (Octin).

**RESULTS.** A large range of concentrations has been tested for each compound. This has been done in order that activity of a particular compound demonstrable only at either high or low concentration would not be overlooked. Since each compound has been subjected to a relatively small number of tests, the data indicate only the qualitative response in particular concentration ranges.

In table 1 are listed the responses of the isolated frog's heart to compounds which may be considered as modifications of the basic structure, 2-phenylethylamine. On the basis of this series of amines containing cyclic substituents separated from the amino group by two carbon atoms, certain generalizations concerning the structural requisites for producing the positive inotropic action may be made. All compounds containing the 3,4-dihydroxyphenyl group produce this action on the frog's heart (compounds No. 15-21). Norepinephrine, or arterenol, may be added to these dihydroxyphenyl compounds which stimulate the heart. West (28) reported that *dl*-norepinephrine is eight times as potent as *l*-epinephrine on the isolated frog's heart. The other substances which elicited this response had one hydroxyl group on the benzene ring in either the 3- or the 4- position and a side chain of only two carbon atoms (compounds No. 6-8, 13 and 14). When the amino group is converted to a methylamino, isopropylamino, or even a dimethylamino group, the positive inotropic action remains. However, substances with one hydroxyl ring substituent are inactive if the side chain is lengthened as in the beta-phenylisopropylamine derivatives (compounds No. 9-12). Those compounds with unsubstituted phenyl groups possessed no positive inotropic activity (compounds No. 1-4). Thus, tyramine may be considered the





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positive inotropic activity, this pharmacologic response is not solely dependent on the amino group in the molecule. It thus appears that aliphatic amines may exert their positive inotropic action by a mechanism different from that by which epinephrine stimulates the heart.

A real difference in the action of aliphatic amines and epinephrine was seen in experiments in which hearts were either severely hypodynamic or had ceased beating after being perfused for 24 hours. At this time, 2-methylaminoheptane ('Ocnethyl') produced no stimulation, but epinephrine stimulated the hearts quite markedly. A similar observation was made by Jackson (9) who found that a related aliphatic amine has a potent action on a beating heart, but that this substance, unlike epinephrine, does not start a heart which has ceased to beat. On the other hand, both of the drugs are able to overcome the hypodynamic condition produced by either pentobarbital or a solution containing one-half the usual concentration of calcium. Another interesting type of experiment illustrates the difference in mode of action of the aliphatic amine and epinephrine. When a heart is depressed to the point of nearly complete ventricular standstill by  $1 \times 10^{-4}$  2-methylaminoheptane, epinephrine produces rapid recovery of the heart to a normal beat.

**NEGATIVE INOTROPIC EFFECTS.** This action is quite marked in phenylethylamine derivatives with either no ring substituent or with *p*-hydroxy groups. Methyl or ether groups on the ring appear to increase the depressant properties. Compounds with *m*-hydroxy or dihydroxy substituents display no depressant action at the highest concentration tested. Since phenylalanine and tyrosine did not depress the heart, the carboxyl group appears less detrimental to the heart than a methyl group in the same position in the molecule.

The cyclohexylalkylamines and phenylalkylamines possess only negative inotropic activity, and some of them in rather low concentrations.

**ADRENOLYTIC AGENTS AND THE FROG'S HEART.** Since the structures of the active aliphatic amines differ so widely from the required structure for the aromatic compounds with positive inotropic activity, an attempt was made to separate the two types of activity. The 'adrenolytic' activities of the ergot alkaloids do not extend to their action on the heart. Tests of ergotamine, ergotoxine, and dihydroergotamine confirmed the fact that these substances do not block the action of epinephrine on the heart. The report of Schnetz and Fluch (6) on the inability of Priscol to block the action of epinephrine on the frog heart was also confirmed. Priscol depressed the heart at concentrations above  $1 \times 10^{-5}$ . When complete standstill was produced by 1:10,000 Priscol, epinephrine started the heart and caused a prolonged stimulation. On the mammalian heart, the action of epinephrine is not blocked by dibenamine (7). After contact for a half-hour, with concentrations of dibenamine as high as 1:100,000, the response of the frog's heart to epinephrine is not markedly diminished. Another compound, 2-(piperidinomethyl)-6-methoxy tetralone hydrochloride, which Randall (8) has found to be adrenolytic, is unable to block the action of epinephrine on the frog's heart.

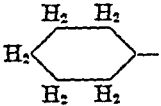

**DISCUSSION.** The earlier studies of the action of amines on the frog's heart have been reviewed by Trendelenberg (10). Nakamura (11) using the Straub preparation observed positive inotropic action in response to adrenalone

*o*-dihydroxyphenyl compounds with 3-carbon side chains. Other reports have appeared on the sympathomimetic properties of *l*-dihydroxyphenylalanine (DOPA). After an intravenous administration into the cat, Holtz and Credner (3) observed a pressor effect. Page and Reed (4) noted the pressor activity of DOPA in the rat. The compound causes glycogenolysis in the perfused toad liver (Horimi, 5).

In table 2 are listed other amines which were studied. Compounds with a cyclohexane ring (compounds No. 25 and 26) or an unsubstituted phenyl ring (compounds No. 27-31) were inactive. There was no positive inotropic activity

TABLE 2

*The action of cyclohexyl- and phenyl-alkylamines and aliphatic amines on the isolated frog's heart*

COMP. NO.	CHEMICAL STRUCTURE		DILUTION			
	Ring	Side Chain	$1 \times 10^{-7}$	$1 \times 10^{-6}$	$1 \times 10^{-5}$	$1 \times 10^{-4}$
25 26		$\text{CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$ $\text{CH}_2\text{CH}(\text{NHCH}_3)\text{CH}_3$		0 0	— 0	— —
27 28 29 30 31		$\text{CH}_2\text{NH}_2$ $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ $\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$ $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$ $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$	  0 0	0 0 0 0 0	0 0 — — —	—* — — — —
32 33 34 35 36 37		$\text{HOCH}_2\text{CH}_2\text{NH}_2$ $\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{NH}_2$ $\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}(\text{NH}_2)\text{CH}_3$ $\text{CH}_3(\text{CH}_2)_4\text{CH}(\text{NH}_2)\text{CH}_3$ $\text{CH}_3(\text{CH}_2)_4\text{CH}(\text{NHCH}_3)\text{CH}_3$ $(\text{CH}_3)_2\text{C}=\text{CH}(\text{CH}_2)_2\text{CH}(\text{NHCH}_3)\text{CH}_3$	   + + +	0 + + + + +	0 + + — +† +†	0* — — — — —

\* Similar action at  $1 \times 10^{-4}$ .

† Irregularities.

observed for any of the phenylalkylamines with the amino group separated by one, or more than two, carbon atoms from the benzene nucleus.

In contradistinction to the above series of amines possessing cyclic groups, the alkylamines (compounds No. 33-37) which were tested were quite potent in producing sustained positive inotropic activity. Only ethanolamine (compound No. 32) proved to be inactive. The active aliphatic amines were rather difficult to wash out of the heart. This property of the aliphatic amines produces a type of tachyphylaxis. The group listed in table 2 includes compounds with the amino group at positions 1- or 2- in the carbon chain, primary and secondary amines, and straight and branched chain compounds.

The only structural resemblance between these active aliphatic compounds and the active epinephrine-like compounds of table 1 resides in the amino group. Since the cyclohexyl- and phenyl-alkylamines listed in table 2 are lacking in

positive inotropic activity, this pharmacologic response is not solely dependent on the amino group in the molecule. It thus appears that aliphatic amines may exert their positive inotropic action by a mechanism different from that by which epinephrine stimulates the heart.

A real difference in the action of aliphatic amines and epinephrine was seen in experiments in which hearts were either severely hypodynamic or had ceased beating after being perfused for 24 hours. At this time, 2-methylaminoheptane ('Oenethyl') produced no stimulation, but epinephrine stimulated the hearts quite markedly. A similar observation was made by Jackson (9) who found that a related aliphatic amine has a potent action on a beating heart, but that this substance, unlike epinephrine, does not start a heart which has ceased to beat. On the other hand, both of the drugs are able to overcome the hypodynamic condition produced by either pentobarbital or a solution containing one-half the usual concentration of calcium. Another interesting type of experiment illustrates the difference in mode of action of the aliphatic amine and epinephrine. When a heart is depressed to the point of nearly complete ventricular standstill by  $1 \times 10^{-4}$  2-methylaminoheptane, epinephrine produces rapid recovery of the heart to a normal beat.

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(kephrine), nor-adrenalone, and tyramine, as well as to  $\beta$ -aminoacetophenone, phenylethylamine and benzylamine. He also found the same action in response to iso-amylamine and other aliphatic amines. However, his tests of these compounds as vasoconstrictors of the vessels of the hind limbs of the frog before and after ergotoxine led him to conclude that of all the compounds tested only adrenalone, nor-adrenalone, and tyramine acted like epinephrine in that their constrictor effects were inhibited by ergotoxine.

The stimulating action of tyramine on the frog's heart has been reported also by Ransom (12) and Tainter (13). The positive inotropic action of tyramine was observed by Mezey and Staub (14) on electrically stimulated ventricular strips of frog's heart. Sollmann (15) found slight and irregular stimulation of the frog's heart perfused *in situ*, in response to ephedrine, but the usual response was depression. The latter finding was confirmed in the Straub preparation by Amatsu and Kubota (16) and Kreitmar (17). According to Miura (18) both ephedrine and pseudo-ephedrine depress the heart of the intact frog.

Recently, Lands *et al.* (19, 20) in studies on the perfused frog heart *in situ* noted a marked stimulation in response to Isuprel, but no response to N-isopropyl- $\beta$ -hydroxy-p-hydroxyphenylethylamine. The potent effect of isopropylepinephrine on the isolated heart also has been reported by Lissak *et al.* (21). It was noted by Swanson and Chen (22) that 2-methylamino-1-cyclopentyl-propane, a pressor amine, has no stimulating action on the frog's heart perfused *in situ*.

A possible explanation for the few positive effects of sympathomimetic agents reported in the literature but not observed in this study may be in order. These agents are known to potentiate epinephrine. Epinephrine-like substances have been extracted from frog hearts (see Euler, 23, in reference to his own work as well as to the work of previous investigators). This may be present in hearts perfused *in situ* or in freshly isolated hearts, but the neurohumor may be removed after prolonged isolation and repeated washings of the heart.

Although ethanolamine has no pressor action (Tainter, 24), it possesses positive inotropic activity when tested on the mammalian heart (Hauschild, 25; Krayner *et al.*, 26). Homma (27) found that 1:4,000 ethanolamine produces a very small positive inotropic action on the frog's heart. We have not seen this action except in tests made without neutralizing the base, and then only in high concentrations.

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#### SUMMARY

1. The isolated frog's heart shows epinephrine-like responses to 2-phenylethylamine derivatives with at least one hydroxyl group in either the 3- or 4- position

on the ring. Compounds with secondary (methyl- or isopropylamino) or tertiary amine groups are also active.

2. When the side chain is lengthened to three carbon atoms, only 3,4-dihydroxyphenyl compounds are active. *l*-Dihydroxyphenylalanine (DOPA) produces positive inotropic effects.

3. The aliphatic sympathomimetic amines produce positive inotropic changes which are reversible only with difficulty. Certain characteristics of the actions of these compounds indicate that the mechanism of action differs from that of epinephrine.

4. Phenyl- and cyclohexyl-alkylamines do not possess positive inotropic activity.

5. Ergot alkaloids, Priscol, 2-(piperidinomethyl)-6-methoxy tetralone and dibenamine are unsatisfactory adrenolytic agents when applied to the frog's heart.

#### REFERENCES

1. HOFFMANN, F., HOFFMANN, E. J., MIDDLETON, S., AND TALESNIK, J.: *Am. J. Physiol.*, **144**: 189, 1945.
2. KRAYER, O., MENDEZ, R., DE ESPANÉS, E. M., AND LINSTAD, R. P.: *THIS JOURNAL*, **74**: 372, 1942.
3. HOLTZ, P., AND CREDNER, K.: *Arch. exper. Path. Pharmacol.*, **20**: 356, 1942-3.
4. PAGE, E. W., AND REED, R.: *Am. J. Physiol.*, **143**: 122, 1945.
5. HORIMI, J.: *Japan. J. Med. Sci. IV, Pharmacol.* **7**, *Proc. Japan. Pharmacol. Soc.*, **113**, 1933. *Chem. Abs.*, **29**: 2236, 1935.
6. SCHNETZ, H., AND FLUCH, M.: *Zeitschr. f. Klin. Med.*, **137**: 667, 1940.
7. NICKERSON, M., AND GOODMAN, L. S.: *THIS JOURNAL*, **89**: 167, 1947.
8. RANDALL, L. O.: Personal Communication.
9. JACKSON, D. E.: *Anesth. and Analg.*, **26**: 3, 1947.
10. TRENDLENBERG, P.: *Hefter's Handbuch der Experimentellen Pharmakologie*, **2**: 1130, 1924.
11. NAKAMURA, M.: *Tohoku J. Exper. Med.*, **6**: 367, 1925.
12. RANSOM, F.: *THIS JOURNAL*, **14**: 367, 1920.
13. TAINTER, M. L.: *THIS JOURNAL*, **30**: 163, 1926-7.
14. MEZEY, K., AND STAUB, H.: *Arch. exper. Path. Pharmacol.*, **182**: 183, 1936.
15. SOLLMAN, T.: *THIS JOURNAL*, **30**: 21, 1936.
16. AMATSU, H., AND KUBOTA, S.: *Kyoto Igaku Zasshi* **14**, No. 1, 77, 1917. *Japanese Medical Literature*, **2**, No. 5, 2, 1917. *Chem. Abs.* **12**: 2019, 1917.
17. KREITMAR, H.: *Arch. exper. Path. Pharmacol.*, **120**: 189, 1927.
18. MIURA, K.: *Berlin. Klin. Wchnschr.*, **24**: 707, 1887.
19. LANDS, A. M., RICHARDS, E. E., NASH, V. L., AND HOOPER, K. Z.: *THIS JOURNAL*, **89**: 297, 1947.
20. LANDS, A. M., NASH, V. L., MCCARTHY, H. M., GRANGER, H. R., AND DERTINGER, B. L.: *THIS JOURNAL*, **90**: 110, 1947.
21. LISSÁK, K., MARTIN, J., AND ÁNGYÁN, A.: *Orvosok Lapja Népegészségügy*, **2**: 129, 1946. *Chem. Abs.*, **42**: 3074, 1948.
22. SWANSON, E. E., AND CHEN, K. K.: *THIS JOURNAL*, **93**: 423, 1948.
23. EULER, U. S. V.: *Acta Physiol. Scand.*, **12**: 73, 1946.
24. TAINTER, M. L.: *Arch. Internat. Pharmacodyn. et de Therap.*, **46**: 192, 1933.
25. HAUSCHILD, F.: *Arch. exper. Path. Pharmacol.*, **201**: 569, 1943.
26. KRAYER, O., FARAH, A., AND UHLE, F. C.: *THIS JOURNAL*, **88**: 277, 1946.
27. HOMMA, R.: *Tohoku J. Exper. Med.*, **24**: 167, 1934.
28. WEST, G. B.: *J. Physiol.*, **106**: 418, 1947.

# INOTROPIC SYNERGISM OF CARDIAC GLUCOSIDE WITH CALCIUM ACTING ON THE FROG'S HEART IN ARTIFICIAL MEDIA<sup>1</sup>

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The relation of calcium ion concentration to the effect of cardiac glucosides has been studied by many investigators, among them Loewi (1); Lloyd (2); Kanschegg (3); La Barre and van Heerswynghe (4); Smith, Winkler and Hoff (5); and Baker (6). A high proportion of these studies have concerned the poisoning of intact animals, without regard to inotropic (i.e., "therapeutic") action as related to the concentration of the applied glucoside. In addition, however, the hypocalcic heart under appropriate conditions affords a useful means of evaluating the "therapeutic" effectiveness of a cardiac glucoside in restoring the contraction of such a hypodynamic frog heart. It is the purpose of this report to formulate quantitatively the relationship of 1) the percentile contractile response of the myocardium referred to maximal concentration; 2) the concentration of calcium ion; and 3) the concentration of cardiac glucoside.

**METHODS. Procedure.** The technique employed was based upon that used by McLean and Hastings (7). Male frogs (*Rana pipiens*) weighing about 30 gm. were used. The bicarbonate-phosphate buffered Krebs-Henseleit solution (8) employed as a medium had the following composition:

0.9 per cent NaCl	3.82 per cent $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	Solution saturated with 95
1.15 per cent KCl	1.30 per cent $\text{NaHCO}_3$	per cent $\text{O}_2$ and 5 per cent
2.11 per cent $\text{KH}_2\text{PO}_4$	Glucose, 123 mgm./100 cc.	$\text{CO}_2$ mixture

For convenience throughout this report the above variety of Ringer-Locke's solution so constituted has been referred to simply as "Locke's solution".

The test organ was the isolated frog heart prepared according to Straub (9), but with the modified cannula described by Kraye, Linstead and Todd (10). When filled to a height of 4 cm., such a cannula held approximately 2 cc. of solution. For simple changes of  $\text{Ca}^{++}$  ion concentration, 1 cc. of the new solution was added and at once removed with a finely tipped pipette after five or six beats of the heart; then the full amount of serum or Locke's solution was added up to the desired pressure-head mark on the cannula. When a change of medium was involved, or when a change in glucoside concentration was made, three preliminary equilibrations were employed at five-minute intervals. Thereafter the definitive series of calcium concentrations was studied at four minute intervals: so that the crucial effect on the 50-percentile contraction was reached not earlier than 40 minutes after the first intro-

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duction of the new ouabain. The final reading was obtained after four minutes. The record was made by a camera on a continuous strip of photo-sensitive paper from a bright light source reflected off a small mirror rigidly fixed to a torsion spring, as described in a previous communication from this laboratory (11). The final records gave a maximal excursion of nearly 10 cm., and represented a magnification of about 40-fold.

The data of the present experiments comprise a series of contractile responses, each corresponding to a known (or estimated) concentration of calcium ions. Each of these calcium-response curves was conducted at a known concentration of ouabain or other glucoside. In general, the concentration of free ouabain employed ranged from 0 to 6 micrograms per cent in Locke's solution. The temperature ranged from 22° to 23° C.

In all experiments due time was allowed to permit the heart to come to equilibrium with the ouabain (or digitoxin) of the medium, e.g., over 35 minutes for ouabain and over 50 for digitoxin. Under these precautions experiments were performed to study the influence of a series of increasing glucoside-concentrations.

*Analysis of Data.* From nearly 50 experiments, each about ten hours in length with readings taken at four-minute intervals, it is possible to report only selected representative observations. In describing the results algebraically, the chief variables to be considered are the following:

- 1) the percentile contractile response,  $R$ , of the myocardium;
- 2) the concentration of calcium ion,  $[Ca^{++}]$ ; and
- 3) the concentration of cardiac glucoside,  $[G]$ .

*The Selection of Intercept  $[Ca_{50}^{++}]$  vs.  $[Ca_{50}^{++}]$ .* In accordance with the traditional use of the  $ED_{50}$ , it would be desirable to express the experimental results in terms of  $[Ca_{50}^{++}]$ . Forsooth, as described in the companion paper to be published soon (12), this intercept is satisfactory because the algebra is simplified and the center of statistical weight of the data lies close to the 50-percentile response,  $R_{50}$ . In Locke's solution, however, the heart is liable to injury at low concentrations of calcium. Hence it is undesirable to depress the cardiac response (in per cent) much below the  $R_{50}$  value. Consequently, the latter value is slightly more accurate. In practice, appropriate constants are used for either, but the difference is trivial. For the sake of clarity, the results are expressed here in terms of the  $[Ca_{50}^{++}]$ , but in actual experiments the authors recommend the use of the  $[Ca_{50}^{++}]$ .

*Observations in Locke's Solution* (based on  $[Ca_{50}^{++}]$ ). As shown in table 1 and figure 1, the data of Experiment 25 yield a series of calcium-response curves, which can be fitted satisfactorily to straight lines when calcium ion is plotted against  $\log (90-R)$ . These lines intersect the ordinate for 50-percentile contraction at regular intervals. Consequently a linear relationship can be formulated between each glucoside concentration  $[G]$  and the intercept delineated by the corresponding line.

This point is demonstrated more readily when calcium ion is plotted against  $\left(\frac{R}{100-R}\right)$ , as shown in figure 2. At the 50-percentile level, i.e., at the ordinate marked 1.0, the curves for contraction form a series of regular intercepts. By a fortunate coincidence of units, the ordinate of figure 2 may be used again to indicate the concentration of hydrated ouabain. When the several intercepts for 50-percentile contraction are projected on to the appropriate ordinates, another nearly linear relationship results according to the following equation:

$$\text{Equation A} \quad [Ca_{50}^{++}] = A - k[G]$$

where the calcium is expressed in millimols per liter and the concentration of ouabain in micrograms per cent. In this experiment,  $k$  has the value 0.93. Ob-



TABLE 1  
Calcium-ouabain synergism in Locke's solution (experiment 25) at 27°C.

CONCENTRATION OF OUBAIN (HYDRATED) VS. CARDIAC CONTRACTILE RESPONSE (PERCENTILE)†																								
[Ca <sup>++</sup> ] mMols.	0 microgm. % Oubain				1 microgm. % Oubain				2 microgm. % Oubain				3 microgm. % Oubain				4 microgm. % Oubain				5 microgm. % Oubain			
	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$	$r_1 \times 10$ mm.	%	$\frac{R}{100-R}$	$\log \frac{(90-R)}{(100-R)}$
	52†				34†				32.6†				26.6†				26.5†				27†			
2.0	52	100			34	100			32.6	100			26.6	100			26.5	100			27	100		
1.8	40	77			30	89			30	92			26	99			25	94.5						
1.5	40	77			32	94			30	92			28	105			21	89						
1.3	36	69							33	101			26	99			26	99						
1.2					28	92.5	4.7																	
1.0	36	99*	2.2	1.32	25	73.5*	2.8	1.22	32	98			23	86.5			24	90.5						
0.8	32	91.5*	1.76	1.45	23	67.7*	2.05	1.35	28	67*	2.0	1.36	20	75*	3.0	1.18	19	72*	2.6	1.26				
0.7	25	47*	0.9	1.63	21	62*	1.6	1.45	23	70*	2.3	1.39					29	75*	3.0	1.18	22	81.5*	4.4	1.09
0.6	24	45.5*	0.8	1.65	13	38*	0.62	1.72	18	55*	1.6	1.54	17	64*	1.78	1.41	19	72*	2.6	1.26	20	74*	2.85	1.20
0.5	22	42			9	23.5*	0.31	1.82	11	34*	0.52	1.75	15	59.5*	1.3	1.53	17	64*	1.78	1.41	20	74*	2.85	1.20
0.4	18	35			1	3.4			13	40*	0.7	1.70	7	26.2*	.37	1.89	17	64*	1.78	1.41	18	67*	2.0	1.36
0.3	10	31							1	3.6			1	3			10	41.5*	.75	1.69	17	63*	1.65	1.43
0.2	5	9.6															3	11.3			3	11		
Av. $\left( \frac{R}{100-R} \right)$			1.42				2.01				1.42				1.61				2.09					2.34
[Ca <sup>++</sup> ]	0.71(8)				0.65(2)				0.54(6)				0.48(3)				0.37(3)					0.23(9)		
[Ca <sup>++</sup> ]	0.81(7)				0.75(1)				0.64(7)				0.57(8)				0.45(3)					0.30(3)		

\* These data used in calculations.

† Note that, as defined in the text  $R$  is a percentile response, whereas  $r$  is an observed contraction.

† Maximum contraction.

viously,  $A$  is the value for  $[Ca_{50}^{++}]$  in Locke's solution free of ouabain, i.e., 0.71. It will be noted that in table 1 the mid-response has been calculated both for the 60- and the 50-percentile values. Obviously, Modified Equation A

$$[Ca_{50}^{++}] = (A - a) - k [G] = A' - k [G]$$

where  $A'$  equals 0.81.

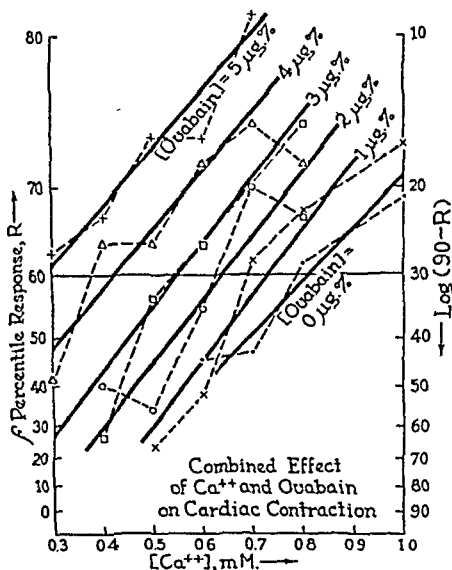


FIGURE 1. The contractile response,  $R$ , of the frog's heart at various concentrations of ouabain can be plotted conveniently on a reverse-logarithmic scale. This function is labelled  $f$  on the ordinate. Because conventions vary in different journals, it should be emphasized that the right hand ordinate gives arithmetic values plotted on a log scale. The calcium concentration, however, is linear on the abscissa.

**Mathematical Formulation.** It is apparent from figure 2 that the  $[Ca_{50}^{++}]$  serves as a common denominator through which the response of the heart can be translated into the concentration of ouabain employed. When each of the two last-named variables has been related to the value for  $[Ca_{50}^{++}]$  common to both, the calcium may then be eliminated from the picture. There results the relationship shown in figure 3, in which the constant  $A$  now appears as the intercept on the  $y$ -axis. In this figure, three functions of the glucoside concentration are plotted on

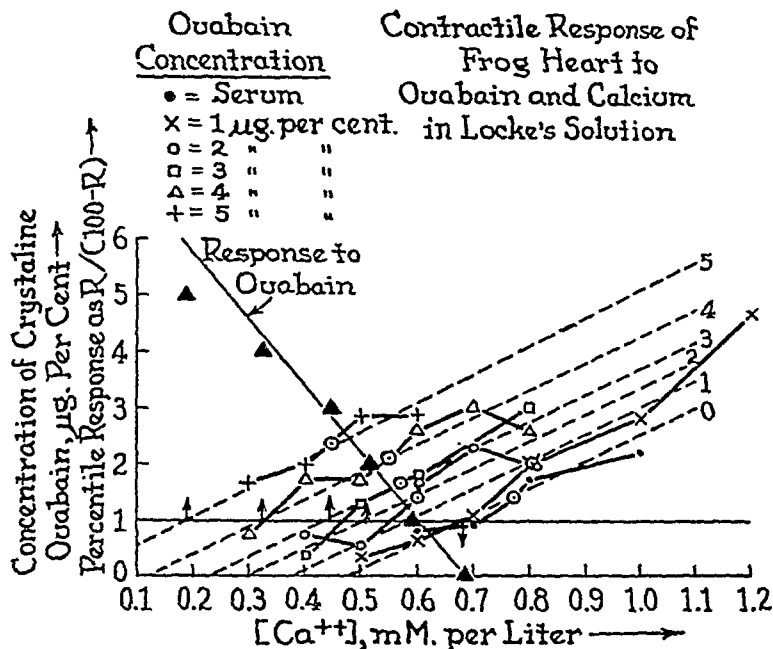


FIG. 2. In this graph the ordinate is used twice. With calcium as the independent variable, the contractile response,  $R$ , of the frog's heart gives the 50-percentile response at a value of 1.0 (i.e., 50:50) for each concentration of hydrated ouabain. Then each 50-percentile response is projected (see arrows) to the appropriate value for ouabain concentration. The synergistic relationship of ouabain to calcium is shown to be approximately linear.

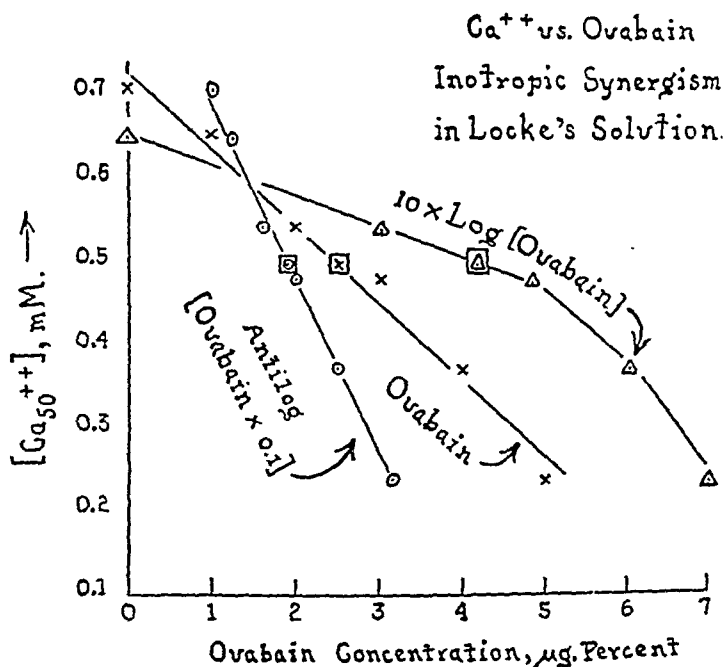


FIGURE 3. Although traditionally one might expect the log-concentration to be the determining function, it is clear that the simple concentration of ouabain (or even the anti-log) better describes the relationship to calcium ions.

the  $x$ -axis, i.e., (a) the log-concentration, (b) the direct concentration, and (c) the antilog-concentration. It will be noted that the direct concentration serves satisfactorily to describe the experimental data, and thus illustrates Equation A already given.

For those investigators who would prefer to calculate the concentration of glucoside directly from the data illustrated in figures 1 and 2, the following amplifications of Equation A will be convenient. Figure 1 yields the equation

Equation B

$$0.76 \{ \log (90-50) - \log (90-R) \} = k [G] + [Ca^{++}] - A$$

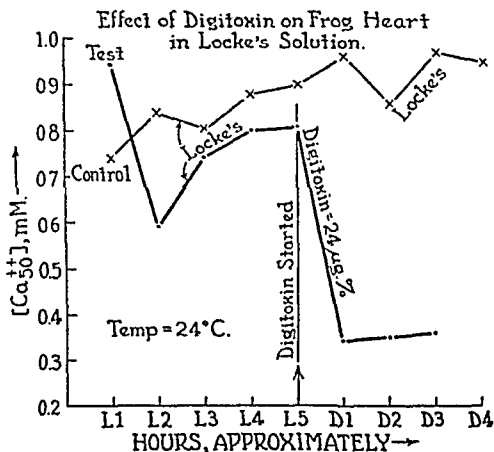


FIGURE 4. In contrast to the behavior of the control heart, the frog heart treated with digitoxin shows a prompt decline in calcium requirement.

Likewise figure 2 yields

$$\text{Equation C} \quad 1/b \left( \frac{R}{100 - R} - 1 \right) = k[G] + [Ca^{++}] - A$$

where  $1/b$  equals 1.8.

*Other Cardiac Glucosides.* The technique, as illustrated for hydrated ouabain, may also be applied to other cardiac glucosides. In figure 4 is demonstrated the effect of a high (near-toxic) concentration of crystalline digitoxin upon the  $[Ca_{50}^{++}]$ . The rather prompt response is in striking contrast to the data for a control heart. Likewise, in figure 5, are shown comparative data for hydrated ouabain and for digitoxin, respectively. By appropriate calculation these data yield an equivalence value of over three units of digitoxin per unit of hydrated ouabain. Obviously, it is not the purpose of the present report to present detailed assays

of various cardiac glucosides. The principle, however, is illustrated by figure 5. The method might be of value in the screening of prospective cardiac drugs.

DISCUSSION. The mechanism of calcium effects on the contractile response of the myocardium is *not* the thesis of this communication. In 1928 Clark, Percival and Stewart (13) suggested that a readily dissociable calcium-lipoid complex located at the *surface* of the individual cell might be the reason for the rapid adjustment to changes in calcium ions. By way of contrast, it should be noted that an appreciable time is required to produce the full inotropic effect of ouabain.

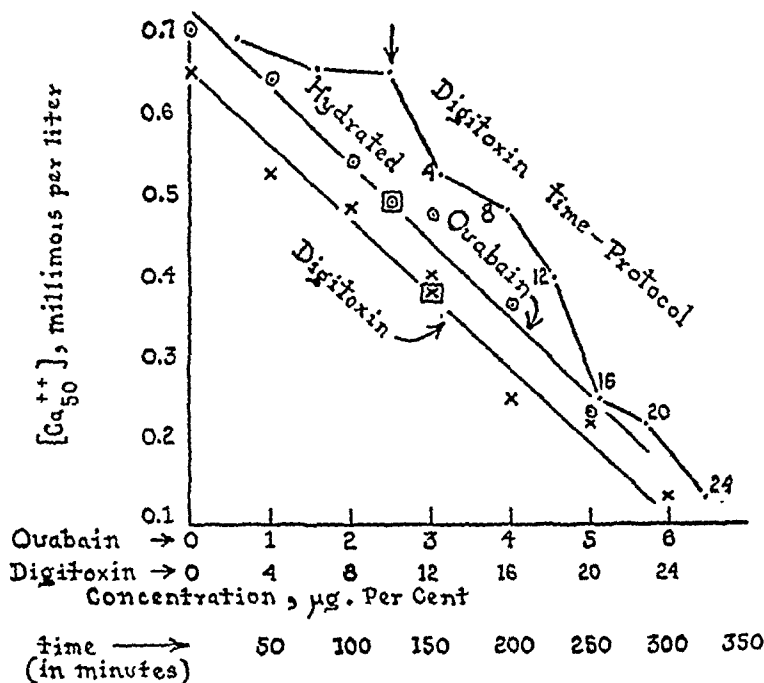


FIGURE 5. Comparative calcium-glucoside responses are shown for ouabain and for digitoxin. The scale for concentrations of digitoxin is arbitrarily selected at four times that of hydrated ouabain. In addition the protocol for the digitoxin experiment is plotted against time to illustrate the experimental procedure.

Whenever calcium ions are reduced in the medium, the contractile response of the heart can be restored by adding a *proportionate* amount of glucoside. In short, each molecule of a given glucoside compensates for a certain loss of calcium, which is characteristic of the drug in question. For instance, at 27° C. as shown in figure 3,

$$\text{Equation } D \quad \frac{\Delta[\text{Ca}_{50}^{++}]}{\Delta[\text{G}]} = -6400$$

for hydrated ouabain, when both concentrations are expressed in molality.

It should be emphasized that the effects of ouabain described here are inotropic,—not toxic. Many studies of toxic arrhythmia, A-V block, or myocardial

standstill at high concentrations of drug have been published, both in intact animals or men and in surviving organs. Such toxic effects were reviewed by Smith, Winkler and Hoff (5), who studied the toxic synergism with calcium in dogs. Baker (6) has made carefully systematic studies of toxic dosage for intact rabbits and of toxic concentrations in isolated (Langendorff) rabbit hearts. He found that increased calcium or decreased potassium shortened the survival time and *vice versa*. In our studies thus far, potassium has been maintained constant. Recently Bine and Friedman (14, 15) have used the embryonic duck heart to record arrhythmia due to a cardiac glucoside. Their values fall in the general range described in this report, although calcium ions were not varied and the dosage of glucoside was often higher than the truly inotropic.

In general, the classic experiments of Trendelenburg (16) on the effects of calcium deficiency in body fluids have been confirmed by the control data presented in this report.

#### SUMMARY

The isolated frog heart responds to concentrations of hydrated ouabain about 5 micrograms per cent (i.e., 0.0001 millimolar) in Locke's solution. Thus  $p$  [Ouabain] is about 7 in the inotropic range, like  $pH$  and  $p$  [Thiamine]. When the isotonic contraction of the heart is depressed by lowering the concentration of calcium ions, the contractile response may be restored by adding ouabain or digitoxin. This synergism follows simple linear functions relating  $k$  [G] to  $[Ca_{10}^{++}]$ , where  $k$  is the relative potency of the drug and [G] the concentration of the drug. This technical approach can be applied to other cardiac drugs; so that potency is measured in terms of the number of calcium ions for which each molecule of drug can compensate.

#### REFERENCES

1. LOEWI, O.: Arch. f. exper. Path. u. Pharmacol., 82: 131, 1917-1918.
2. LLOYD, W. D. M.: Brit. Med. J., 1: 662, 1928.
3. KONSCHIEG, A.: Arch. f. exper. Path. u. Pharmacol., 71: 251, 1913.
4. LABARRE, J., AND VAN HEERSWYNGHELS, J.: Arch. internat. de pharmacodyn. et de therap., 61: 233, 1939.
5. SMITH, P. K., WINKLER, A. W., AND HOFF, H. E.: Arch. Int. Med., 64: 322, 1939.
6. BAKER, J. B. E.: Brit. J. Pharmacol., 2: 259, 1947.
7. McLEAN, F. C., AND HASTINGS, A. B.: J. Biol. Chem., 107: 337, 1934; HASTINGS, A. B., McLEAN, F. C., EICHELBERGER, L., HALL, J. L., AND DA COSTA, E.: J. Biol. Chem., 107: 351, 1934.
8. KREBS, H. A., AND HENSELEIT, K.: Ztschr. f. physiol. Chem., 210: 33, 1932.
9. STRAUB, W.: Verhandl. Ges. deutsch. Naturforsch. u. Aerzt., 84: 191, 1912.
10. KRAYE, O., LINSTAD, R. P., AND TODD, D.: THIS JOURNAL, 77: 113, 1943.
11. WHITE, W. F., AND SALTER, W. T.: THIS JOURNAL, 88: 1, 1946.
12. SALTER, W. T., SCIARINI, L. J., AND RUBIN, B.: To be published.
13. CLARK, A. J., PERCIVAL, G. H., AND STEWART, C. P.: J. Physiol., 66: 346, 1928.
14. FRIEDMAN, M., AND BINE, R., JR.: Proc. Soc. Exper. Biol. and Med., 64: 162, 194, 1947.
15. BINE, R., AND FRIEDMAN, M.: Am. J. Med. Sci., 216: 534, 1948; FRIEDMAN, M., AND BINE, R., JR.: Am. J. Med. Sci., 214: 633, 1947.
16. TRENDLENBURG, P., UND GOEBEL, W.: Arch. f. exper. Path. u. Pharmacol., 89: 171, 1921.

# A COMPARISON OF THE EFFECTS OF ETHER AND CYCLOPROPANE ANESTHESIA ON THE RENAL FUNCTION OF MAN

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**INTRODUCTION.** The effects of anesthesia upon renal function have been studied recently by several investigators (1-4). The previous studies in human subjects employing ether and cyclopropane have been conducted during surgical procedures. It has been impossible to separate changes influenced by operation from those caused by the anesthetic itself. We report here the results of measurement of renal function just prior to and during ether and cyclopropane anesthesia in normal humans. No surgery was undertaken before completing the observations.

**METHODS.** Mannitol and sodium para-aminohippurate were used for measurement of glomerular filtration rate and effective renal plasma flow, respectively<sup>2</sup>. The quantities given, methods of administration, collection of blood and urine samples, and methods of analysis were essentially those recommended by Goldring and Chasis (5). The only difference was that we found it necessary to give substantially less sodium para-aminohippurate in both the priming and sustaining infusions than they recommend in order to insure low plasma levels of this substance.

**PLAN OF STUDY.** An attempt was made to exclude complicating factors that produce a divergence from homeostasis except those caused by the anesthetic agent itself. The patients chosen were those in whom elective surgical procedures were anticipated. They were in good clinical condition, and had normal kidney function by the usual rough clinical criteria. It must be emphasized that all studies were completed before any surgical procedures were started.

Twenty to thirty minutes after the sustaining solution had been started, three ten-minute control periods were completed. At least two blood samples were drawn for every three urine collection periods<sup>3</sup>. Usually, no premedication had been given, and some patients were rather apprehensive. Hence, the effect of excitement on renal function cannot be excluded during the pre-anesthetic stage. During induction and until the patient had reached the first to second plane (Guedel) of the third stage of anesthesia, clearance measurements were interrupted. Collections were then started again and continued at approximately ten-minute intervals for an additional 23 to 74 minutes. Anesthesia was maintained insofar as possible at these same levels throughout the period of observation. Eight patients received ether, and seven received cyclopropane.

**RESULTS.** *Ether* (tables 1, 3). Four males and four females between the ages of 19 and 62 were studied. In two, nitrous oxide was used briefly as an induction agent (J. B. and J. V.); in the remaining six, no supplementary anesthesia was employed. Collection periods during anesthesia were started 14 to 30 minutes after induction and continued for 29 to 74 minutes more.

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<sup>2</sup> We are indebted to Dr. William P. Boger of Sharpe and Dohme, Inc., for the generous supply of mannitol and sodium para-aminohippurate used in these studies.

<sup>3</sup> All urine collections were made through indwelling multiple eye catheters.

TABLE I  
*Ether*

PATIENT	AGE	SEX	AVERAGE GLOMERULAR FILTRATION CC./MIN./1.73 sq. m.	AVERAGE RENAL PLASMA FLOW CC./MIN./1.73 sq. m.	AVERAGE URINE VOLUME CC./MIN.	AVERAGE MANNITOL U/F RATIO	BLOOD PRESSURE MM. HG	PLANE OF THIRD STAGE ANESTHESIA	DURATION OF ANESTHESIA	
									Before Renal Study	Duration Renal Study
									Before	During
J. B.	35	F	128 117	573 685	2.5 1.8	13 56	128/80 113/70	Upper 2	22	41
R. M.	19	F	118 96	751 452	2.5 1.4	41 59	108/56 122/52	Upper 2	30	45
H. M.	31	M	135 93	835 481	3.8 1.6	37 58	122/70 145/62	Lower 1	25	39
J. V.	31	M	72 46	578 251	3.1 1.6	21 33	110/70 128/65	Upper 2	21	74
V. T.	62	M	100 78	466 293	4.5 2.3	24 36	108/60 89/50	Lower 2	14	32
S. C.	37	F	87 76	380 264	6.1 1.7	14 45	120/70 125/68	Mid 2	20	30
D. I.	49	M	83 70	424 265	2.3 2.3	39 33	125/85 133/73	Upper 1	26	35
M. C.	38	F	95 68	543 200	3.2 1.2	32 62	140/80 165/85	Lower 1	29	29
Mean ..			102 ±8	569 ±56	3.5 ±0.5	32 ±3.6	110/70	Upper 2		
Standard error of mean...			81 ±8	319 ±19	1.7 ±0.1	48 ±1.1	135/75	Lower 1		



The glomerular filtration rates, comparing the average values determined during control periods with those determined during anesthesia, fell in every patient. The average reduction in all patients was 21 per cent. Certain variations and chronological trends not apparent from the mean values should be noted. In one patient, J. B., filtration rate fell early in anesthesia, although probably still within the normal range, and in subsequent periods returned to the control level. In another, J. V., filtration rate fell markedly and persisted at lower levels throughout the period of observation. The changes in the remaining six patients varied between the extremes seen in these two. The usual response was an early significant fall followed by slight improvement in filtration rate as anesthesia continued. In three patients (J. V., S. C., D. I.), the filtration rates during the control periods were below normal. This suggested the presence of possible underlying renal disease not revealed by the usual clinical methods. However, they showed the same general trends and quantitative changes in filtration rate during ether anesthesia as demonstrated in the group as a whole.

With one exception, J. B., average effective renal plasma flow fell during ether anesthesia; the average decrement for all patients was 39 per cent. Like filtration rate, there were individual variations not apparent from mean values in the response of plasma flow to ether. In J. B., plasma flow after an initial fall then rose above the control values, whereas in V. T., it was persistently decreased. This latter effect was essentially that seen in the remaining six patients, although in some there appeared to be a tendency after an initial fall for the plasma flow to improve towards the control levels.

The average filtration fraction ( $C_M/C_{PAH}$ ) rose in seven of the eight patients studied; hence, the plasma flow was reduced proportionately to a greater degree than was the filtration rate. The average increase for all patients was 25 per cent.

Average urine flow diminished significantly in all but one patient, D. I., and the average decrement for the group was -51 per cent. The mannitol U/P ratio rose in the seven patients whose urine volume diminished, indicating that the diminished urine flow was a result of an increase in renal tubular reabsorption of water as well as of the decrease in glomerular filtration rates and effective plasma flow.

There were no striking changes in the blood pressure.

*Cyclopropane* (tables 2, 3). Two males and five females between the ages of 16 and 41 were studied. Nitrous oxide was used briefly as an induction agent in three patients (R. J., C. F., M. S.). Collection periods during anesthesia were started 8 to 18 minutes after induction and continued for 23 to 41 minutes more. The changes occurring during cyclopropane anesthesia were qualitatively similar to but quantitatively greater than those found with ether.

The average glomerular filtration rate fell in all instances during anesthesia and to a significant degree in all except one patient, C. F. The average decrement for all cases was 31 per cent, as compared with 21 per cent for ether. The usual response was a significant and persistent lowering in glomerular filtration rate. As with the ether patients, examination of individual protocols revealed some

TABLE 2  
*Cyclopropane*

PATIENT	AGE	SEX	AVERAGE GLOMERULAR FILTRATION CC./MIN. / 1.73 SQ. M.		AVERAGE RENAL PLASMA FLOW CC./MIN. / 1.73 SQ. M.		AVERAGE URINE VOLUME CC./MIN.		AVERAGE MANNITOL U/P RATIO		BLOOD PRESSURE MM. HG.		PLANE OF THIRD STAGE ANESTHESIA	DURATION OF ANESTHESIA	
			Anesthesia		Anesthesia		Anesthesia		Anesthesia		Anesthesia			Before Renal Study	Duration Renal Study
			Before	During	Before	During	Before	During	Before	During					
G. D.	16	M	106	79	730	380	3.0	1.8	34	48	120/80	164/50	Upper 2	10	26
R. J.	17	M	104	85	624	107	5.2	2.1	20	41	120/80	166/79	Mid 2	12	34
C. F.	11	F	99	95	733	388	3.2	1.5	29	58	110/70	138/62	Upper 2	18	23
M. S.	22	F	104	71	535	228	2.9	1.1	33	41	120/80	152/72	Lower 1	11	41
L. D.	35	F	77	53	517	210	2.0	2.0	35	30	105/80	130/92	Upper 2	8	39
I. G.	40	F	87	41	471	181	3.2	1.5	32	34	110/70	121/77	Upper 3	18	27
O. G.	27	F	113	51	535	150	2.8	1.1	40	18	130/70	145/98	Lower 2	15	37
											130/85	152/93	Mid 2		
													Lower 2		
Mean.....			99	68	503	282	3.2	1.7	32	43					
Standard error of mean....			±5	±8	±39	±39	±0.4	±0.1	±2.3	±3.6					

temporal variations in this function during cyclopropane anesthesia not demonstrable from mean values. In L. D., for example, after an initial sharp fall, the filtration rate returned to control levels.

Effective renal plasma flow also fell in each instance, 52 per cent if all cases are averaged, as compared with 39 per cent for ether. In a few subjects, plasma flow improved slightly as anesthesia continued, but, in general, it was strikingly and persistently diminished throughout the duration of cyclopropane anesthesia.

Here, as with ether, the reduction of effective plasma flow was relatively greater than that of glomerular filtration rate as indicated by a rise in filtration fraction in all patients. The average rise for the entire group was 35 per cent, as compared with 25 per cent with ether.

Average urine flow during cyclopropane was diminished slightly less than during ether, and average U/P ratio increase was slightly less than with ether. Individual variations, however, were present. In one patient, urine flow was unchanged. In six patients, urine flow decreased, accompanied in five of these by a significant rise in mannitol U/P ratio. In I. G., the U/P ratio was essentially unchanged, while urine flow and filtration rate during anesthesia were approximately half those observed during control periods. Here, presumably, there was no increase in tubular reabsorption of water. Both L. D. and I. G. during the

TABLE 3

ANESTHESIA	NO. PATIENTS	AVERAGE CHANGES (PER CENT)			
		$C_M$	$C_{PAH}$	$C_M/C_{PAH}$	Urine flow
Ether.....	8	-21	-39	+25	-51
Cyclopropane.....	7	-31	-52	+35	-47

control periods showed filtration rates well under the normal range; hence, their failure to show an increase in U/P ratio might be related to intrinsic renal disease, although there was no clinical evidence of such in either patient.

The systolic blood pressure rose in four patients, and was unchanged in three. The diastolic pressure fell in two of the four with systolic elevation, rose in one, and was unchanged in one. The diastolic pressure fell in one patient whose systolic pressure did not change.

DISCUSSION. Our findings are in some disagreement with those of other workers. Previous studies in man demonstrated no changes in inulin or diodrast clearances with ether anesthesia, and a fall in inulin clearance and variable diodrast clearance changes with cyclopropane (1). These studies differed from ours in that they were complicated by the probable effects of operation on renal function. In dogs under light anesthesia with both ether and cyclopropane, little change in either glomerular filtration rate or effective renal plasma flow occurred (2). With both drugs under deep anesthesia (deeper presumably than we employed in any of our observations), urine flow, glomerular filtration rate, renal blood flow, and tubular reabsorption of glucose were reduced to about half the values observed during light anesthesia. Similar studies in dogs, made with sodium pentobarbital, revealed no important changes in renal function (3).

In our series some changes in renal function, as demonstrated by diminished glomerular filtration rate and effective plasma flow and increased filtration fraction, were observed in all but one subject with ether, and in all subjects with cyclopropane. The effects of cyclopropane were more marked and persistent than those of ether.

The levels of anesthesia maintained (first to second plane of the third stage) are those employed for most surgical procedures in this institution.

The rise in filtration fraction in our subjects deserves special mention. It was not observed in the previous studies already cited (1, 2); yet it occurred in the majority of our patients, and it was more marked with cyclopropane than with ether. In reference to this, the studies of Craig *et al.* show some similarities. In their dogs (2, table 3, p. 114), the filtration fraction under cyclopropane was significantly higher than with ether, but it is true that the filtration fraction with both agents fell as the animals passed from light to deeper anesthesia.

Although we have no extensive data concerning the importance of extra-renal factors, those we do have would render unlikely a conclusion that alterations in homeostasis played any important part in the changes observed. All of our patients were adequately hydrated prior to the period of observation, and there was no clinical evidence of dehydration or decrease in plasma volume during anesthesia. Changes in blood pressure were usually slight and apparently unrelated to the observed changes in renal function.

The observed facts from our measurements are most consistent, we believe, with the hypothesis that ether and cyclopropane, acting either primarily upon the kidney and its blood vessels or secondarily by a humoral mechanism, cause renal vasoconstriction of both the afferent and efferent arterioles, but that they have a greater relative action on the efferent arterioles. While qualitatively the two drugs have similar effects, quantitatively those of cyclopropane are more pronounced. It seems unlikely that the changes observed can be explained by the production of renal vascular shunts. Angiographic studies in rabbits during prolonged anesthesia with sodium pentobarbital and ether failed to show any definite change in the caliber of the renal vessels (6). The reduction in urine volume observed in all but one patient, and the usual rise in mannitol U/P indicate release of antidiuretic hormone in addition to the changes in renal hemodynamics.

Effective blood flow in the kidney appears to be reduced more greatly by cyclopropane than by ether. A similar effect has been observed in two other regions of the body.

Beecher, Warren and Murphy (7) have found when cervical lymph is collected, under standardized conditions, that on shifting an average dog from cyclopropane to ether, the lymph flow increases 63 per cent. On shifting an average dog from ether to cyclopropane, the lymph flow is reduced 35 per cent. The lymph flow is lower in all cases under cyclopropane than it is under ether. There can be little question under the circumstances of these observations that the lymph flow follows the capillary circulation.

Zweifach, Hershey, Rovenstine and Chambers (8) have studied the blood flow in the capillary bed of the dog's omentum during graded hemorrhage. This they

have done under several anesthetic agents. They have observed directly that as bleeding continues under cyclopropane, the number of open capillaries remains normal for about a half-hour, with constriction persisting for the following three hours. During this period the flow persists through the most direct channels, the "thoroughfare channels". But with ether, under the same circumstances, the number of open capillaries remains normal for one and one-half hours (three times as long as with cyclopropane). With ether the normal period is followed by a relatively brief (45-minute) constrictive phase (one-quarter as long as with cyclopropane), and then an unrestricted condition follows with widespread flow through all capillaries under ether but not under cyclopropane.

Thus, information derived from three entirely different approaches indicates that the capillary blood flow is reduced by cyclopropane in comparison with ether.

Certain therapeutic implications are suggested from these data. During traumatic shock, alterations in renal function similar to those here reported have been well demonstrated (9). If shock is severe or prolonged, these changes may persist for some time after resuscitation has been effected, and in some instances death from uremia results (10, 11). These previous studies demonstrated no such residual effects of ether anesthesia itself in the absence of shock (cyclopropane was not studied). Consideration must be given to the possibility that administration of the anesthetic agent while shock is present could accentuate further deviations from normal renal function. Judging from the observations reported here, ether is preferable to cyclopropane in patients if shock is present or anticipated during operation.

#### SUMMARY

1. The renal clearances of mannitol and sodium para-aminohippurate were determined before and during anesthesia. Ether was administered to eight human subjects and cyclopropane to seven.

2. All studies were conducted on patients in good condition with normal renal function as inferred from the usual clinical criteria. All observations were completed before any surgical procedures were started.

3. In seven of the eight patients who received ether, and in all who received cyclopropane, the average glomerular filtration rate and effective renal plasma flow fell during anesthesia. The average filtration fraction rose in each patient except in one, who also showed no significant changes in mannitol or para-aminohippurate clearances. Average urine flow fell in both groups, accompanied by a rise in the average U/P ratio. Individual variations in all of these measurements were observed.

4. The changes in kidney function observed during ether and cyclopropane anesthesia were qualitatively similar; quantitatively, they were greater with cyclopropane, and somewhat less subject to individual variation. Thus, average glomerular filtration rate fell 21 per cent with ether, and 31 per cent with cyclopropane; average effective renal plasma flow fell 39 per cent with ether and 52

per cent with cyclopropane; average filtration fraction rose 25 per cent with ether and 35 per cent with cyclopropane.

5. Possible reasons for the variation of our data from that of previous workers are presented. Certain physiologic and therapeutic implications of these data are suggested.

#### REFERENCES

1. COLLIER, F. C., REES, V. L., CAMPBELL, K. N., IOB, V. L., AND MOTER, C. A.: *Ann. Surg.*, **118**: 717, 1943.
2. CRAIG, F. N., VISSCHER, F. E., AND HOUCK, C. R.: *Am. J. Physiol.*, **143**: 103, 1945.
3. CORCORAN, A. C., AND PAGE, I. H.: *Am. J. Physiol.*, **140**: 231, 1943.
4. SMITH, H. W., ROVENSTINE, E. A., GOLDRING, W., CHASIS, H., AND RANGES, H. A.: *J. Clin. Investigat.*, **18**: 319, 1939.
5. GOLDRING, W., AND CHASIS, H.: Hypertension. The Commonwealth Fund, 1944.
6. TRUETA, J., BARCLAY, A. E., DANIEL, P. M., FRANKLIN, K. J., AND PRICHARD, M. M. L.: *Studies of the Renal Circulation*. Charles C. Thomas, 1947.
7. BEECHER, H. K., WARREN, M. F., AND MURPHY, A. J.: *Am. J. Physiol.*, **154**: 475, 1948.
8. ZWEIFACH, B. W., HERSHEY, S. G., ROVENSTINE, E. A., AND CHAMBERS, R.: *Proc. Soc. Exper. Biol. & Med.*, **56**: 73, 1944.
9. LUTSON, H. D., BRADLEY, S. E., COURNAND, A., AND ANDREWS, V. V.: *J. Clin. Investigat.*, **23**: 381, 1944.
10. BURNETT, C. H., SHAPIRO, S. L., SIMEONE, F. A., BEECHER, H. K., MALLORY, T. B., AND SULLIVAN, E. R.: *Surgery*, **22**: 856, 1947.
11. BURNETT, C. H., SHAPIRO, S. L., SIMEONE, F. A., BEECHER, H. K., MALLORY, T. B., AND SULLIVAN, E. R.: *Surgery*, **22**: 991, 1947.

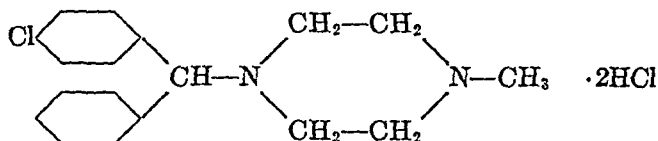
# A PHARMACOLOGICAL STUDY OF N-METHYL-N'-(4-CHLOROBENZHYDRYL) PIPERAZINE DIHYDRO-CHLORIDE—A NEW ANTIHISTAMINIC

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The finding in these laboratories that some substituted piperazines possess definite antihistamine activity, has led to the development of a new class of highly potent antihistaminics of chemical structure distinctly different from the usual ethylenediamine types which are in the market. The most promising member of this new class is N-methyl-N'-(4-chlorobenzhydryl) piperazine dihydrochloride<sup>1</sup> (Compound 47-282), for which the generic name Chlorcyclizine<sup>2</sup> has been adopted.



It is the purpose of this communication to point out some of the steps that led to the development of Chlorcyclizine and to present the results of preliminary pharmacological studies. For the sake of comparison, the results obtained with known antihistaminic drugs are also given.

**ANTIHISTAMINIC ACTIVITY. Tracheal Chain.** As it has been pointed out in previous communications (1-3), the guinea pig tracheal chain responds to histamine with prompt contractions which are sustained at the same level for a long period of time, thus facilitating the qualitative and quantitative study of the ability of the antihistamine drug to relieve the spasm. Therefore, as illustrated in figure 1, the technic we use is that which estimates the degree of relaxation caused by the addition of the antihistamine drug to the bath at the peak of the histamine contraction, allowing the antihistaminic to act until the maximum relaxation is obtained.

In table 1 are listed the antihistamine activities of some of the substituted piperazines which led to the development of Chlorcyclizine. The respective potencies are given in terms of diphenhydramine (Benadryl) taken as 100 per cent.

Although the N-methyl-N'-benzyl piperazine (46-125) was found to have some antihistamine activity, the substitution of an ethyl or the methyl group (46-126)

<sup>1</sup> It has recently come to our attention that this compound, in the form of the monohydrochloride, has been independently synthesized and tested for antihistamine activity at the Abbott Laboratories, North Chicago, Illinois.

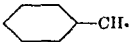
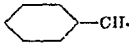
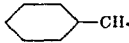
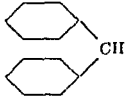
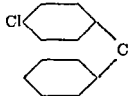
<sup>2</sup> Supplied as 'Perazil' Brand Chlorcyclizine by Burroughs Wellcome & Co. (U.S.A.) Inc., Tuckahoe, New York.

yielded a compound of less activity. The N-benzyl-N'-normal lauryl piperazine (895) was found to be devoid of activity. Therefore, it appeared that the group attached to R' had to be light, preferably methyl. On the other hand, when a heavier group was attached to R (in the case of 47-83 a benzhydryl), a highly active antihistamine compound, comparable to Benadryl, was obtained.

The incorporation of a chlorine in the 4 position of one of the phenyl groups of the benzhydryl piperazine yielded Chloreyclizine which, as shown in table 1 and figure 1, was found to be four times more potent than Benadryl. As illustrated

TABLE 1

*The antihistaminic activity of the piperazines as determined on the guinea pig tracheal chain*

COMPO NO	$\begin{array}{c} \text{CH}_2-\text{CH}_2 \\ \diagup \quad \diagdown \\ \text{R} \quad \quad \text{R}' \\ \diagdown \quad \diagup \\ \text{CH}_2-\text{CH}_2 \end{array} \quad 2\text{HCl}$		ANTIHISTAMINE POTENCY IN TERMS OF DIPHENHYDRAMINE TAKEN AS 100%
	R	R'	
46 125		CH <sub>3</sub>	Slight
46 126		CH <sub>2</sub> -CH <sub>3</sub>	Very slight
895		n-C <sub>11</sub> -H <sub>23</sub>	None
47 83		CH <sub>3</sub>	100%
Chloreyclizine		CH <sub>3</sub>	400%

in figure 1, Chloreyclizine acted more slowly than 47-83 and Benadryl, but its antihistaminic effect after washing persisted for a longer period of time.

*Protection Against Nebulized Histamine*—The method of I oew (4) as modified by Siegmund (5) was also used to test for antihistaminic activity. This method possesses the advantage of revealing the oral effectiveness of the drug. Guinea pigs, one at a time, were confined in a glass chamber of about four liters capacity and exposed to a finely atomized mist of a 0.2 per cent solution of histamine diphosphate. Only those guinea pigs suffering asphyxial collapse within four minutes were accepted for the test. In our experience guinea pigs become increasingly sensitive to repeated tests within the same day and it is rare that such animals can withstand a six minute exposure. Therefore any guinea pig surviving six minutes' exposure to the nebulized histamine was arbitrarily classed as "protected", and



in the following experiments such protection was attributed to the drug. In carrying out the actual tests, the acceptable guinea pigs were allowed to recover from the screening procedure for about one hour. At the end of this time they were dosed orally with the drugs

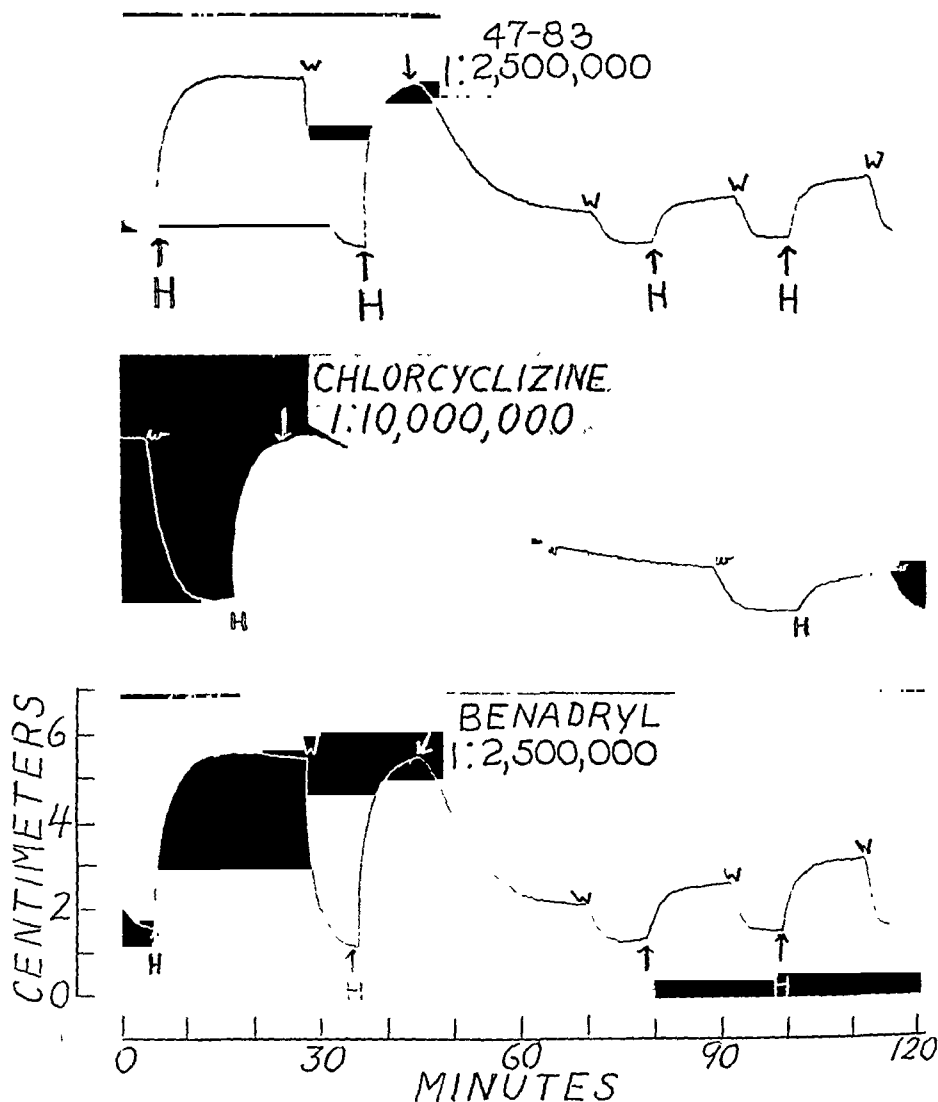


FIG. 1. The effect of the indicated concentrations of 47-83, Chlorcyclizine and Benadryl on the spasms induced by (H) histamine phosphate 1:250,000 on the guinea pig tracheal chain. (W) indicates washing.

under examination. One hour later the animals were subjected to the nebulized histamine. This procedure was repeated at 2½, 4 and 5 hours after dosing. All drugs were given at the same dosage level of 2.5 mgm. per kgm. although it is recognized that some may be more potent than others. The data are given in table 2.

The results indicate that Chlorcyclizine is an effective antihistaminic when given orally to guinea pigs, and has an activity comparable to commercially available, highly potent, antihistaminic drugs

The duration of the action of Chlorcyclizine in protecting guinea pigs against histamine aerosol, was determined in two groups of ten animals each. As shown in table 3, a single oral dose of 10 mgm per kgm. was found to impart marked protection for over 23 hours, and some residual activity was still present at 43 hours.

*Inhibition of Histamine-Induced Spasm of the Guinea Pig Ileum* As figure 2 shows, Chlorcyclizine like Benadryl possesses the ability to inhibit the contractile action of histamine on the isolated guinea pig ileum. At a dilution of 1:50,000,-

TABLE 2

*The effect of Chlorcyclizine and other antihistaminics on experimental asthma produced in guinea pigs by exposure to nebulized histamine*

DRUG	ORAL DOSE  mgm /kgm	NUMBER OF PIGS DOSED	PER CENT GUINEA PIGS PROTECTED (HOURS AFTER DOSING)			
			1 hr	2½ hrs	4 hrs	5 hrs
Chlorcyclizine	2.5	37	62	75	83	69
Pyriminamine (Ncoantergan)	2.5	5	60	60	40	60
Chlorothene (Tagithen)	2.5	8	63	50	63	63
Methapyrilene (Ibenylene)	2.5	5	10	20	20	0
Triphenylamine (Pr benzamine)	2.5	5	60	10	10	20
Controls	—	10	0	0	0	0

TABLE 3

*Duration of action of Chlorcyclizine in protecting guinea pigs against nebulized histamine*

ORAL DOSE  mgm /kgm	NUMBER OF PIGS DOSED	PER CENT GUINEA PIGS PROTECTED (HOURS AFTER DOSING)					
		0.1 rs	1.1 rs	19 hrs	21.1 rs	23 hrs *	43 hrs
10	10	0	80	70	70	50	30
10	10	0	90	—	—	60	10

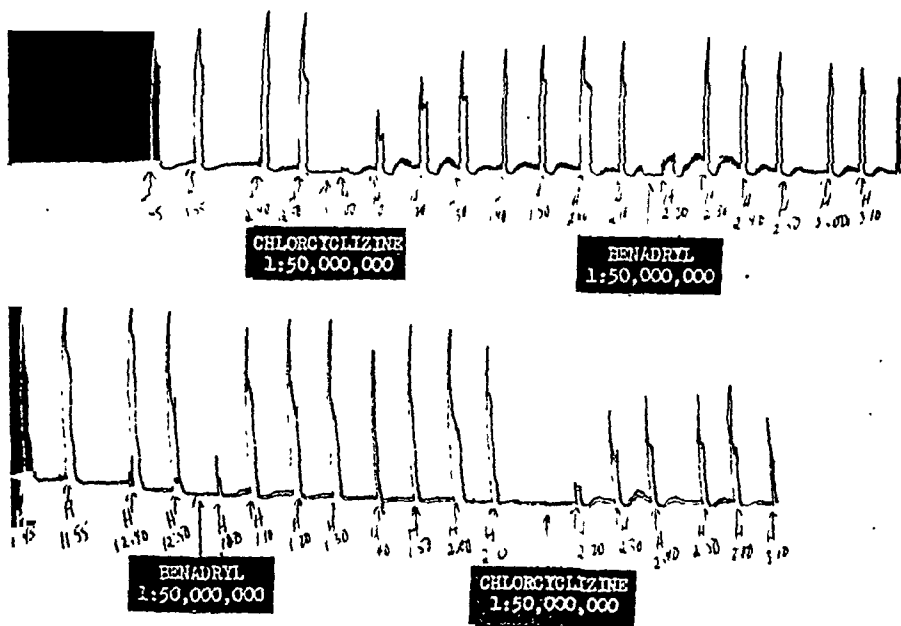
000 both drugs exhibit approximately the same intensity of action, but Chlorcyclizine is more difficult to remove by washing and consequently displays a longer duration of action.

*Inhibition of the Vasodilator Action of Histamine on Dog's Blood Pressure* Three mgm per kgm of Chlorcyclizine injected intravenously in dogs under Dial anesthesia, blocked almost completely for over two hours the depressor effect produced by a dose of 50 microgm of histamine diphosphate.

*Inhibition of Histamine Wheal Response in Humans* Unpublished data (6) obtained from experiments on 30 subjects show that a single oral dose of 100 mgm. of Chlorcyclizine modifies for over 24 hours the reaction produced by the topical application of a histamine solution to small scarified areas in the skin.

**Clinical Observations.** Preliminary clinical trials (7) indicate that single daily doses of 50 mgm. of Chlorcyclizine are very effective in relieving common allergic symptoms. A very low incidence of side-effects is reported by the patients.

**SPASMOLYTIC ACTION.** Chlorcyclizine was found to be effective not only in antagonizing the spasms produced by histamine on the isolated guinea pig ileum, but also was found to inhibit the spasmogenic effects of acetylcholine and barium chloride. It was found to be least effective in antagonizing barium chloride, moderately active against acetylcholine, but extremely effective in antagonizing histamine.



found that the duration of anesthesia produced by the injection of a 1 per cent solution of Chlorcyclizine was about the same as that produced by an equal concentration of Benadryl, both lasting about 22 minutes

**MYDRIATIC TESTS** One per cent solution of Chlorcyclizine had no mydriatic action on the rabbit eye. The same concentration of Benadryl produced a 20 per cent dilation of the pupil, which could be ascribed to the atropine like action of this drug. Therefore, Chlorcyclizine is relatively free from action of this character

TABLE 4

*The acute toxicities of Chlorcyclizine and known antihistaminics when injected intraperitoneally in white mice*

DRUG	LD <sub>50</sub>	LIMITS OF ERROR
	mgm /kgm	Per cent
Chlorcyclizine	137	95-105
Pyranisamine (Ncountergran)	115	93-107
Chlorothen (Tagathen)	105	92-109
Methapyrilene (Thenvlene)	77	94-108
Tripeleennamine (Pyribenzamine)	67	96-104
Diphenhydramine (Benadryl)	69	93-108
Diatrin	117	96-105

TABLE 5

*Comparison of the tolerance of Chlorcyclizine and Pyribenzamine in albino rats with respect to growth rate and mortality*

CONCENTRATION OF DRUG IN FOOD	CHLORCYCLIZINE			TRIPLEPENNAMINE		
	Average drug consumed mgm /kgm per day	Average weight gain gm. per rat in 55 days	Mortality	Average drug consumed mgm /kgm per day	Average weight gain gm. per rat in 55 days	Mortality
Per cent			Per cent			Per cent
Control	0	171	0	—	—	—
0.00625	4.1	156	0	3.9	170	0
0.0125	7.8	175	0	8.2	145	0
0.025	17.0	143	0	15.1	163	16.6
0.05	34.3	145	0	32.4	153	0
0.1	65.6	123	16.6	61.3	129	0

**TOXICITY Mice** The acute toxicity of Chlorcyclizine was determined by intraperitoneal injection in white mice. The results are summarized in table 4. For the sake of comparison, the LD<sub>50</sub> values obtained for other antihistaminics are also listed.

The symptoms observed prior to death in the mice injected with Chlorcyclizine were indistinguishable from those produced by Benadryl and tripeleennamine (Pyribenzamine). These consisted of initial excitation, tremors, convulsions, respiratory failure and finally cardiac arrest. All deaths occurred within two

# PHARMACOLOGICAL ACTIONS OF TETRAETHYLPYROPHOSPHATE AND HEXAETHYLTETRAPHOSPHATE

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## INTRODUCTION

The condensed alkylphosphates, hexaethyltetrphosphate (HETP) and tetraethylpyrophosphate (TEPP) were introduced in Germany during the war as insecticides (1, 2). The toxic actions of these substances in rats have been described and they have been shown to be powerful anticholinesterases *in vitro* (3, 4, 5). The effects of HETP on circulatory and other systems and its potentiation of the actions of acetylcholine have been studied by Dayrit, Manny and Seevers (6).

We have studied the effects of HETP and TEPP on various systems in the chloralosed cat and on isolated organ preparations. A preliminary account of these actions has been presented (7).

**METHODS.** *Cat Experiments.* These were performed under chloralose anaesthesia (0.08 gm./kgm.). Arterial B.P. was recorded in the common carotid artery. A tracing of the respiration was obtained by recording the movements of the anterior abdominal wall.

Nerve stimulation was carried out with an electronic square wave stimulator. Except where otherwise stated the pulse width was 2 m.sec. and the intensity of stimulation was maintained at a constant supramaximal value in each experiment.

Salivary secretion was measured by means of a cannula in the submaxillary duct connected to a Gaddum drop recorder.

The tone and contractions of the small and large intestines were recorded by means of small balloons connected to water manometers and volume recorders. In the study of responses of the bladder cystometrograms were recorded. A cannula was tied into the urethra and connected to a water manometer and volume recorder. Three cc. of water were introduced (in 5 sec.) at  $\frac{1}{2}$ -1 min. intervals until reflex micturition was produced.

### *Isolated Organ Preparations:*

*Perfused Rabbit Heart:* The Langendorff preparation was used. Drugs were injected into the perfusing fluid.

*Isolated Intestine.* Rabbit duodenum or guinea pig ileum was suspended in Tyrode solution. The drugs were allowed to remain in contact with the gut for 3 min.; the bath was then washed out twice. The drug dosage has been expressed as the final concentration by weight in the bath.

*Frog Rectus Abdominis Preparation.* The drugs were kept in contact with the muscle for 3 min. and then removed by washing three times.

*Rat Phrenic Nerve-Diaphragm Preparation.* This preparation was set up according to the method described by Bülbring (8) except that a spring-loaded lever was used. The volume of the bath was 40 cc. The rate of stimulation of the nerve was once in 10 sec.

*General Effects of HETP and TEPP in cats under chloralose anaesthesia.* The effects of intravenous injection of different single doses of HETP were as follows:—0.05–0.1 mgm./kgm.: There were no detectable effects on any system.

0.2-0.3 mgm./kgm.: Slight bradycardia and a fall in blood pressure of 10-20 mm. Hg developed within five minutes of the injection. 0.4-0.6 mgm./kgm.: Marked slowing of the heart rate, e.g. to half the initial rate, and a fall in B.P. of 50-100 mm. Hg occurred; the B.P. began to recover in 10-20 min. There was marked salivary and bronchial secretion, and transient signs of respiratory stimulation appeared. Bowel movements became excessive and were followed by defecation; micturition also occurred. The pupils were sometimes constricted, sometimes dilated, and where dilatation occurred the nictitating membrane did not appear to be retracted. Muscle fasciculations and minor convulsive movements were seen, and the animal became extremely sensitive to jarring. 0.8-2.0 mgm./kgm.: Violent convulsions appeared within two to three minutes; the visceral effects were greatly enhanced, the bradycardia being very marked, the B.P. falling to about 40 mm. Hg, salivation and bronchial secretion were very profuse, and there were extremely vigorous gut movements and defecation. Death usually occurred in less than one hour.

Larger total amounts of HETP were required to produce the above effects if given in divided doses, e.g. at 15-30 min. intervals.

Atropine (0.2-0.4 mgm./kgm.) greatly reduced or abolished the visceral responses to HETP given in doses up to 2.0 mgm./kgm. but the muscular fasciculations and convulsive movements were not prevented. Atropinised cats frequently survived still larger doses of HETP, though signs of central nervous inhibition, e.g. respiratory depression, were then liable to occur.

TEPP produced effects very similar to those just described for HETP but it was effective in about one-fourth the dose, i.e. TEPP is about four times as potent as HETP.

Triethylorthophosphate and sodium pyrophosphate in doses up to 20 mgm./kgm. produced no systemic effects.

*A. Actions of HETP and TEPP on various systems in the chloralosed cat.*  
*Cardiac response to peripheral vagus nerve stimulation.* In ten experiments the peripheral end of the cut left vagus nerve was stimulated at different frequencies at a constant supramaximal intensity. Figs. 1 and 2 illustrate the results obtained in typical experiments before and after HETP. After HETP (0.57 mgm./kgm.) stimulation of the vagus nerve produced a more marked and more prolonged fall of blood pressure and slowing of the heart rate. Fig. 3 summarizes graphically the effect of HETP on the cardiac inhibition produced by vagal stimulation. The most striking effect is the prolongation of the period before the heart rate returns to normal, the delay being proportional to the dose.

*Potentiation by HETP of cardiac response to injected acetylcholine.* In three cats HETP (0.4 mgm./kgm.) caused a two to four fold potentiation of the bradycardia response to injected acetylcholine.

*Blocking action of large doses of HETP.* After large doses of HETP (2 mgm./kgm.) vagal stimulation had no effect on the heart rate and the depressor effect of small doses of acetylcholine (0.1 mgm.) was abolished. With large doses of acetylcholine (2 mgm.) a small rise of B.P. occurred.

*Action on salivary response to chorda tympani nerve stimulation.* In three ex-

periments the effects of HETP on the salivary secretion induced by stimulation of the chorda tympani at varying frequencies was studied (fig. 4).

After HETP (0.85 mgm./kgm. in divided doses) the rate of secretion in response to stimulation at frequencies below 10 cyc./sec. was increased about threefold. With higher frequencies of stimulation secretion continued after

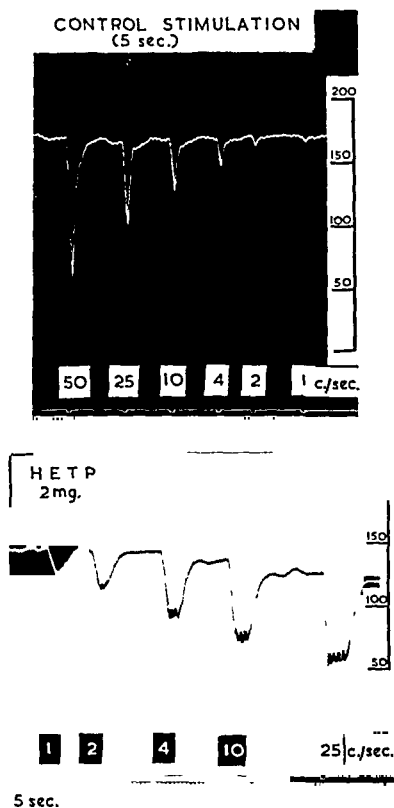


FIG. 1. EFFECT OF HETP ON RESPONSE TO STIMULATION OF PERIPHERAL END OF VAGUS NERVE

Cat, 3.8 kgm., chloralose. Vagi cut.

Record of carotid arterial blood pressure. Scale in mm. Hg.

(a) Control stimulation of left vagus for 5 sec. at frequencies of 50, 25, 10, 4, 2 and 1 cyc./sec.

(b) Stimulation at above frequencies after intravenous injection of 2 mgm. HETP in divided doses. (2 injections of 1 mgm. at 30 min. interval).

Time in 5 sec.

cessation of stimulation. The higher the frequency of stimulation the greater was the volume of this after-secretion. Larger doses of HETP produced a profuse spontaneous secretion. In another experiment (also with the chorda tympani cut) 0.4 mgm./kgm. of TEPP produced a continuous profuse secretion. Stimulation of the peripheral cut end of the chorda tympani produced no further increase in secretion. This might have been due to the fact that maximal secretory activity had already been induced by TEPP alone.

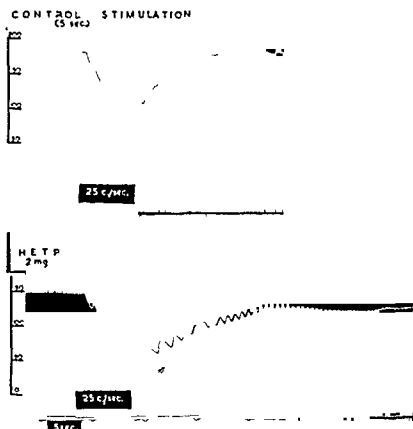


FIG. 2. EFFECT OF HETP ON CARDIAC SLOWING PRODUCED BY STIMULATION OF PERIPHERAL END OF VAGUS NERVE

Cat, 3.6 kgm., chloralose. Vagi cut.

Record of carotid arterial blood pressure. Scale in mm. Hg

(a) Control stimulation for 5 sec. at frequency of 25 c./sec

(b) Stimulation as in (a) after intravenous injection of 2 mgm. HETP in divided doses (2 injections of 1 mgm at 30 min. interval).

Time in 5 sec.

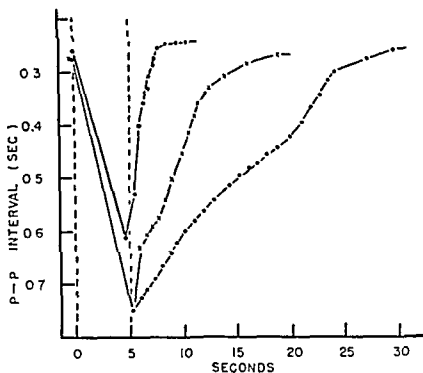


FIG. 3. POTENTIATION BY HETP OF EFFECT ON HEART RATE OF STIMULATION OF PERIPHERAL END OF VAGUS NERVE

Ordinate P-P interval, i.e. interval between successive heart beats, in sec.

Abscissa Time in sec.

Period of stimulation indicated by vertical broken lines. —○—○—Control stimulation for 5 sec. at 25 c./sec —×—×—after 1 mgm. HETP. —●—●—after 2 mgm. HETP



*Actions on intestinal movements.* The actions of HETP on the small intestine and colon were studied in three experiments (fig. 5). Injection of 0.5 mgm./kgm. HETP produced peristaltic intestinal rushes and continuous colonic contractions.

*Action on the bladder.* HETP reduces the distending volume necessary to

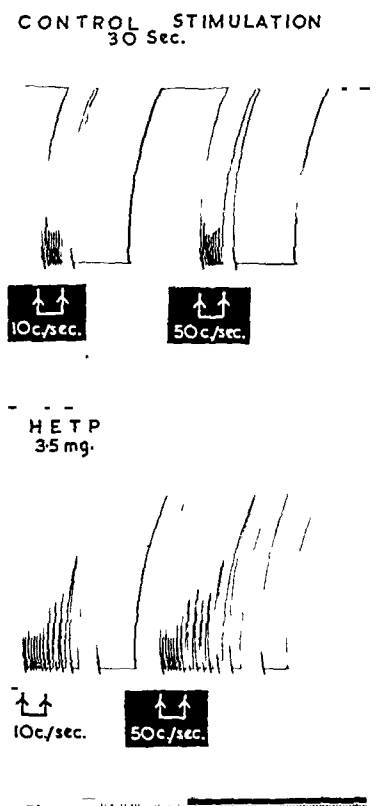


FIG. 4. EFFECT OF HETP ON SALIVARY RESPONSE TO STIMULATION OF CHORDA TYMPANI NERVE

Cat, 4.1 kgm., chloralose.

Record of salivary secretion by Gaddum drop recorder connected to cannula in sub-maxillary duct.

(a) Control stimulation of chorda tympani nerve for 30 sec. at frequencies of 10 cyc./sec. and 50 cyc./sec.

(b) After intravenous injection of 3.5 mgm. HETP in divided doses (initial dose of 1 mgm. followed by 5 injections of 0.5 mgm. at 10 min. intervals). Stimulation of chorda tympani at 10 cyc./sec. and 50 cyc./sec.

Time in 5 sec.

excite the micturition reflex. This effect on the cystometrogram in a typical experiment is shown in fig. 6.

*Effect on the pupil.* Instillation of 1 per cent HETP or TEPP into the conjunctival sac produced marked pupillary constriction within a few minutes (fig. 7). The pupil returned to normal in 24 hours.

*Action on nictitating membrane response to cervical sympathetic nerve stimulation.*

This response was investigated in four cats. Fig. 8 shows the effect of TEPP on the nictitating membrane contraction to stimulation of the cervical sympathetic at various frequencies in the atropinised cat. After injection of 2 mgm./kgm. TEPP the effect of stimulation at 1 cyc./sec. and 2 cyc./sec. was equal to that obtained with control stimulation at five times these frequencies.

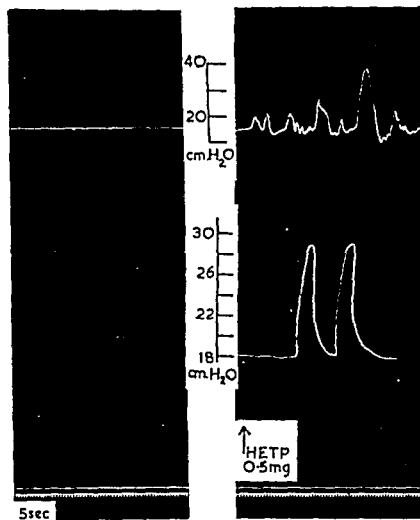


FIG. 5 EFFECT OF HETP ON CONTRACTIONS OF SMALL AND LARGE INTESTINE

Cat 3.7 kgm., chloralose

Balloons in jejunum and colon connected to water manometers and vol. recorders.

Upper record from colon. Lower record from jejunum

(a) Control

(b) Effect of intravenous injection of 0.5 mgm. HETP. Between (a) and (b) 2.5 mgm. HETP were injected in 3 doses of 1 mgm., 1 mgm., and 0.5 mgm. at 5 min. intervals

Time in 5 sec

*Action of TEPP on ganglionic responses to acetylcholine.* The action of TEPP on the ganglionic effects of acetylcholine was studied on the nictitating membrane and the pressor response in atropinised cats. Fig. 9 shows the potentiation of these responses by 4 mgm. and 8 mgm. of TEPP.

*B Action on isolated organ preparations. Rabbit Heart.* The potentiation by TEPP of the action of acetylcholine on the perfused isolated rabbit heart is illustrated in fig. 10. After 40 microgm. of TEPP the inhibitory effect of 0.4 microgm. of acetylcholine was greater and more prolonged than the effect of 4 microgm. acetylcholine before TEPP.

The smallest potentiating dose of TEPP was 4 microgm.; large doses (100-

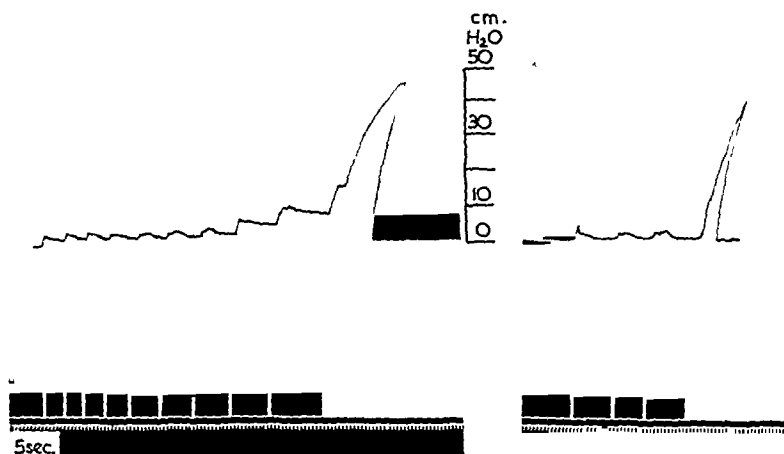


FIG. 6. EFFECT OF HETP ON RESPONSE OF BLADDER TO DISTENTION

Cat, 3.8 kgm., chloralose.

Record of pressure in bladder by cannula inserted through urethra and connected to water manometer and vol. recorder.

(a) Control cystometrogram. At each signal 3 cc. of water introduced into bladder in 5 sec.

(b) Cystometrogram after intravenous injection of 3.5 mgm. HETP in divided doses (initial injection of 1 mgm. followed after 60 min. by 5 injections of 0.5 mgm. at 15 min. intervals).

Calibration cm. water pressure.

Time in 5 sec.



FIG. 7. MIOTIC EFFECT OF HETP

Photograph 30 min. after instillation of one drop of 1 per cent HETP into conjunctival sac of cat's right eye.

500 microgm.) had a marked direct inhibitory action on rate and force of cardiac contraction.

2. *Isolated Intestine Experiments.* *Rabbit duodenum.* HETP in a concentration of  $5 \times 10^{-9}$  increased the tone and the amplitude of contractions and

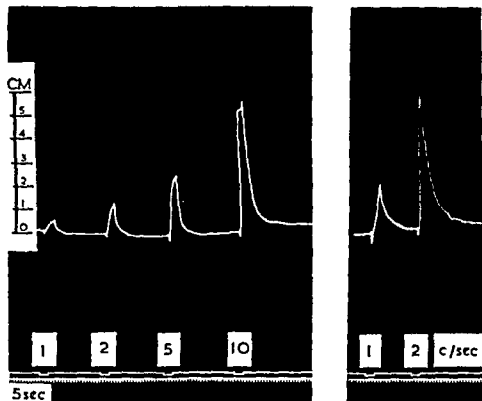


FIG 8 EFFECT OF TLPP ON RESPONSE OF NICTITATING MEMBRANE TO STIMULATION OF CERVICAL SYMPATHETIC NERVE

Cat, 10 kgm, chloralose, atropine (2 mgm)

Record of contraction of nictitating membrane Scale in cm

(a) Control stimulation of cervical sympathetic for 15 sec at frequencies of 1, 2, 5 and 10 c/sec

(b) After intravenous injection of 5 mgm TLPP given in two doses of 1 mgm each at 15 min interval Records taken 2 min after second injection Stimulation of cervical sympathetic for 15 sec at 1 and 2 c/sec

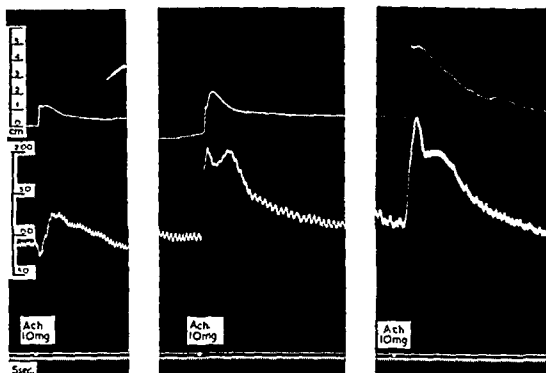


FIG 9 EFFECT OF TLPP ON GANGLIONIC RESPONSES TO INJECTED ACETYLCHOLINE

Cat, 10 kgm, chloralose, atropine (4 mgm)

Upper record contraction of nictitating membrane Scale in cm

Lower record carotid arterial blood pressure Scale in mm Hg

(a) before choline in 3 sec

(c) Two min after a further injection of 1 mgm TLPP At signal injection of 10 mgm acetylcholine in 3 sec

line in 3 sec  
mgm given 20 min  
of 10 mgm acetyl

markedly potentiated the response of the rabbit duodenum to acetylcholine. This effect was annulled by atropine (fig. 11).

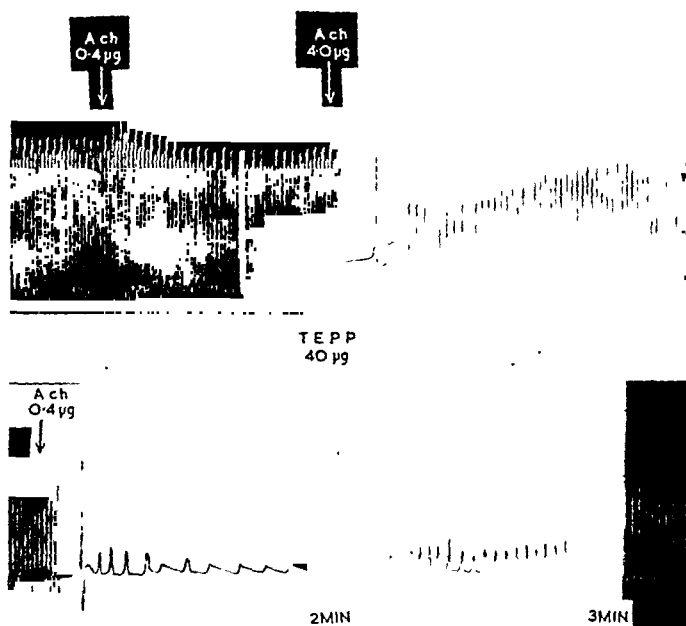


FIG. 10. EFFECT OF TEPP ON PERFUSED RABBIT HEART

Langendorff perfusion of rabbit heart.

(a) Control effect of 0.4 microgm. and 4 microgm. acetylcholine.

(b) After 40 microgm. TEPP. Effect of 0.4 microgm. acetylcholine.

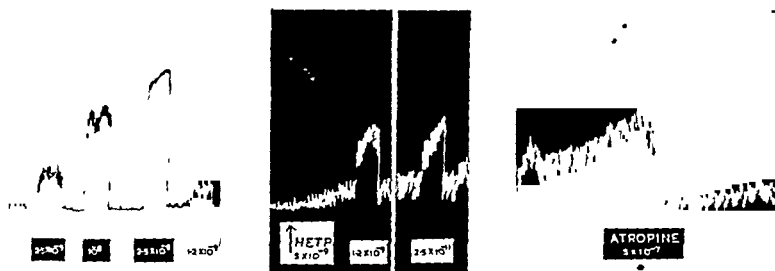


FIG. 11. EFFECTS OF HETP ON CONTRACTIONS OF ISOLATED RABBIT DUODENUM AND RESPONSE TO ACETYLCHOLINE

(a) Control effect of acetylcholine in concentrations of  $1.2 \times 10^{-9}$  to  $2.5 \times 10^{-8}$ .

(b) Effect of HETP ( $5 \times 10^{-9}$ ) followed by  $1.2 \times 10^{-9}$  acetylcholine and after an interval of 5 min.  $2.5 \times 10^{-11}$  acetylcholine.

(c) Continuation of HETP effect. Action of atropine ( $5 \times 10^{-7}$ ).

*Guinea pig ileum.* The potentiation of the action of acetylcholine on guinea pig ileum is illustrated graphically in fig. 12. The curves show the effect of varying concentrations of TEPP on the height of contraction produced by different amounts of acetylcholine. If the amount of acetylcholine required to produce a contraction of 50 mm. is used as a basis for comparison, the extent of

potentiation produced by TEPP is —

	1 times by a concentration of $2.5 \times 10^{-8}$ TEPP
45	" " " " " $5 \times 10^{-8}$ "
200	" " " " " $5 \times 10^{-7}$ "

This potentiation is demonstrated in fig 13 where the logarithms of the potentiation figures are plotted against the concentration of the drug. In this figure are also included data for triethylorthophosphate which is obviously much less potent than TEPP.

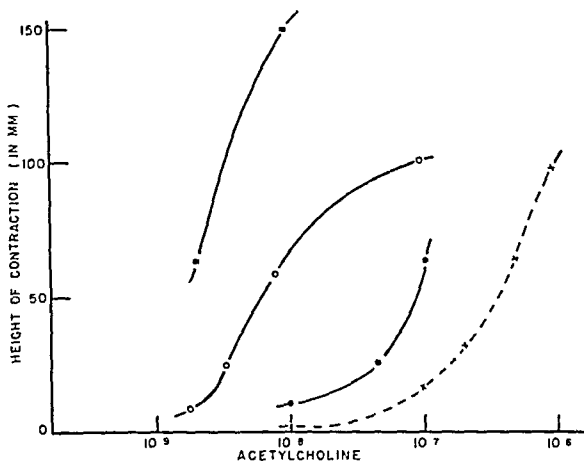


FIG. 12. EFFECT OF TEPP ON THE RESPONSE OF GUINEA PIG ILEUM TO ACETYLCHOLINE

Ordinate: height of contraction in mm  
Abscissa: acetylcholine

—○—○—  $5 \times 10^{-8}$  TEPP  
—■—■—  $5 \times 10^{-7}$  TLPP

*Action on mammalian nerve muscle preparation* The tension of muscular contraction of the rat phrenic nerve diaphragm preparation was increased 60–100 per cent by TEPP in doses of 0.1–1.0 microgm. With larger doses, e.g. 10 microgm, the increase in tension was followed by a rapid decrease.

*Anticuraric action* Fig. 14 shows the antagonistic action of TEPP towards the neuromuscular blocking action of d-tubocurarine Cl.

*Frog Rectus Abdominis preparation* HETP and DFP were compared with respect to direct action, and potentiation of acetylcholine effect, on the frog rectus abdominis preparation. Fig. 15 shows potentiation of the action of acetyl-

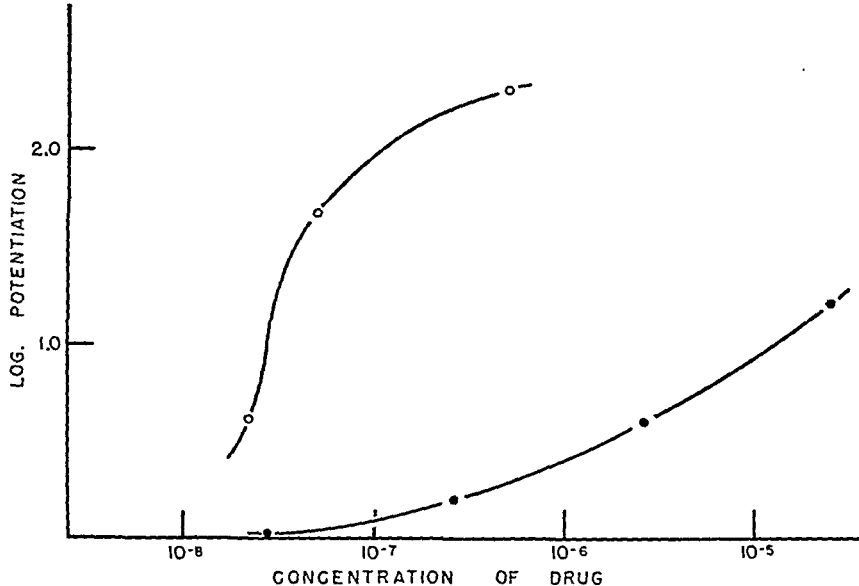


FIG. 13. GRAPHS RELATING THE CONCENTRATION OF TEPP AND TRIETHYLORPHOSPHATE TO THEIR POTENTIATING EFFECT ON THE RESPONSE OF THE ISOLATED GUINEA PIG ILEUM TO ACETYLCHOLINE

Ordinate: log. potentiation.

Abscissa: Concentration of potentiating drug.

—○—○— Tetraethylpyrophosphate.

—●—●— Triethylorthophosphate.



FIG. 14. ANTICURARE EFFECT OF TEPP

Rat phrenic nerve diaphragm preparation. Vol. of bath 40 cc. Supramaximal stimulation at 6/min. Pulse width, 0.5 m.sec.

Effect of (1) 20 microgm. d-tubocurarine chloride (d-Tub.)

(2) 10 microgm. TEPP.

(3) 20 microgm. d-tubocurarine chloride.

choline by different concentrations of HETP. The full curve of DFP action could not be recorded owing to the appearance of a direct stimulation of the muscle in a concentration of  $10^{-4}$ . This stimulation was probably due to the acid reaction of the DFP solution. It should be emphasized that the potentiation of acetylcholine action was smaller when HETP was still in contact with the muscle at the time of acetylcholine application. The potentiation was greatest after two to three washings.

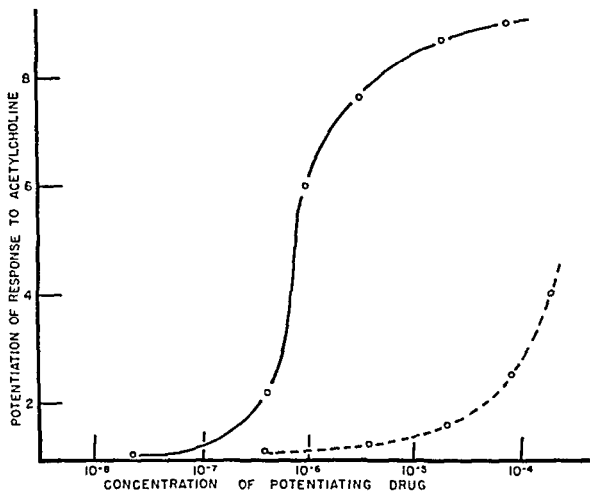


FIG. 15. GRAPHS RELATING THE CONCENTRATION OF HETP AND DFP TO THEIR POTENTIATING EFFECT ON THE RESPONSE OF FROG RECTUS ABDOMINIS MUSCLE TO ACETYLCHOLINE

Ordinate: Potentiation of response to acetylcholine.

Abscissa: Concentration of potentiating drug.

—○—○— HETP.

- - -○- - - DFP.

*Discussion.* Dubois and Mangun (4) and Mangun (5) have shown that *in vitro* both HETP and TEPP are powerful anticholinesterases. These compounds, like eserine, would be expected to preserve acetylcholine at cholinergic nerve endings and to produce actions similar to both the muscarinic and nicotinic effects of acetylcholine (9).

The potentiation of vagal inhibition of the heart by eserine was first shown by Arnstein and Sustschinsky (10) and the similar action of neostigmine has been recorded by Mendez and Ravin (11). We have found that HETP and TEPP produce entirely similar effects and, like eserine and neostigmine, also potentiate the cardiac inhibitory effects of injected acetylcholine.

The potentiation by HETP and TEPP of the salivary response to chorda tympani nerve stimulation was similar to that described by Beznak and Farkas



for eserine (12). In causing stimulation of intestinal muscle in the intact cat, sensitization of the isolated gut to acetylcholine, increased sensitivity of the bladder to distension, and constriction of the pupil on direct application to the eye, HETP and TEPP act just like eserine. These actions of the condensed alkylphosphates are furthermore antagonised by atropine.

The potentiation of the frog rectus abdominis response to acetylcholine, the potentiation (or inhibition in high concentrations) of the rat phrenic nerve-diaphragm response, and the anti-curare actions of HETP and TEPP more closely simulate the actions of eserine than those of DFP.

The potentiation of the nictitating membrane response to stimulation of the cervical sympathetic nerve has never been easy to elicit with eserine, but it was clearly shown with TEPP, which also enhanced the response of the nictitating membrane to injected acetylcholine. The ganglionic action of injected acetylcholine (after atropine) in raising B.P. was also enhanced by HETP, as was the pressor response to central vagus stimulation.

Qualitatively the pharmacological actions of HETP and TEPP bear a close resemblance to those of eserine and neostigmine. The main difference is to be found in the duration of action which is some five to ten times greater for both HETP and TEPP as measured in the unanesthetised rat and in man (13, 14). In potency TEPP seems to be about equal to eserine, and HETP is about one-fourth as powerful.

DFP differs from both these groups of compounds in not potentiating the cardio-depressor action of peripheral vagus stimulation and in producing a much less marked fall of blood pressure; when large doses do produce a bradycardia it is usually due to heart block (15, 16). Further the effects of DFP develop slowly and pass off very slowly indeed. It has been argued that since DFP inactivates cholinesterase irreversibly, the rate of recovery after DFP is dictated by the rate of re-synthesis of cholinesterase. Brauer (17) has shown, however, that TEPP and HETP also produce an irreversible inactivation of cholinesterase and, therefore, if this hypothesis were true one would expect the rate of recovery after all these drugs to be similar. At present no satisfactory explanation can be offered for this discrepancy.

#### SUMMARY

1. The condensed alkylphosphates, hexaethyltetraphosphate (HETP) and tetraethylpyrophosphate (TEPP) are powerful anticholinesterase substances with actions similar to, but more prolonged than, those of eserine and neostigmine (Prostigmin).

2. In the chloralosed cat HETP (0.4–0.6 mgm./kgm.) produced marked bradycardia, fall in blood pressure, marked salivary and bronchial secretion, respiratory stimulation, and increased activity of gut and bladder leading to defecation and micturition. Muscle fasciculations and minor convulsive movements also occurred. Larger doses (0.8–2.0 mgm./kgm.) produced immediate violent convulsions and very profound visceral effects. Death resulted in less than one hour.

3. TEPP acted like HETP but was about four times as potent. Triethylor-

thophosphate and sodium pyrophosphate in doses up to 20 mgm./kgm. produced no systemic effects.

4. Atropine antagonised most of the visceral actions of HETP and TEPP, but did not influence the central nervous or muscular effects.

5. HETP and TEPP sensitised the heart in the anaesthetised cat to the inhibitory actions of vagal stimulation or injection of acetylcholine. They also potentiated the effect of chorda tympani nerve stimulation on salivary secretion.

In atropinised cats these compounds enhanced the effect of cervical sympathetic nerve stimulation on the contraction of the nictitating membrane; they also potentiated the nictitating membrane response and the rise of blood pressure produced by large doses (e.g. 10 mgm.) of acetylcholine.

6. HETP and TEPP potentiated the responses to acetylcholine of the isolated perfused rabbit heart, the isolated rabbit duodenum and guinea pig ileum, and the frog rectus abdominis muscle.

7. TEPP in small concentrations (e.g. 1 in 400 million to 1 in 40 million) increased the force of muscular contraction in the rat phrenic nerve-diaphragm preparation; 1 in 4 million was inhibitory. TEPP antagonised the paralysing effects of d-tubocurarine Cl on this muscle.

8. Intravenous injection of HETP or TEPP usually produced constriction of the pupil, but sometimes dilatation occurred. Direct instillation of the drugs into the conjunctival sac produced marked pupillary constriction, which passed off in about 24 hours.

We are very grateful to Mr. B. Topley for help and advice on chemical aspects of the condensed alkylphosphates, and to Messrs. Albright & Wilson for supplies of materials. We should like to thank Professor S. Wright for his constant encouragement and Mr. F. J. Haydon for technical assistance.

#### REFERENCES

- (1) SCHRADER, G.: B.I.O.S. Final Report No. 714, Item No. 8, 1947.
- (2) LUDVIG, G. F., AND DECKER, G. C.: J. Econ. Entomol., 40: 97, 1947.
- (3) DEICHMAN, W. D., AND WITHERING, S.: Fed. Proc., 6: 322, 1947.
- (4) DUBOIS, K. P., AND MANGUN, G. H.: Proc. Soc. Exper. Biol. and Med., 64: 137, 1947.
- (5) MANGUN, G. H.: Fed. Proc., 6: 353, 1947.
- (6) DAYRIT, C., MANNY, C. H., AND SEEVERS, M. H.: THIS JOURNAL, 92: 173, 1948.
- (7) BURGEN, A. S. V., KEELE, C. A., CHENNELLS, M., DEL CASTILLO, J., FLOYD, W. F., SLOME, D., AND WRIGHT, S.: Nature, 160: 760, 1947.
- (8) BULBRING, E.: Brit. J. Pharmacol., 1: 38, 1946.
- (9) DALE, H. H.: THIS JOURNAL, 6: 152, 1944.
- (10) ARNSTEIN, C., AND SUSTCHINSKY, P.: Central f. d. med. Wissenschaft., Jahrgang V: 625, 1867.
- (11) MENDEZ, R., AND RAVIN, A.: THIS JOURNAL, 72: 80, 1941.
- (12) BEZNAK, M., AND FARKAS, E.: Quart. J. Exper. Physiol., 26: 265, 1936.
- (13) BURGEN, A. S. V.: to be published.
- (14) BURGEN, A. S. V., KEELE, C. A., AND McALPINE, D.: Lancet, 1: 519, 1948.
- (15) MODELL, W., KROP, S., HITCHCOCK, P., AND RIEKER, W. F.: THIS JOURNAL, 87: 400, 1946.
- (16) HEYMANS, C., AND JACOBS, J.: Arch. internat. Pharmacodyn. et de Therap., 74: 233, 1947.
- (17) BRAUER, R. W.: THIS JOURNAL, 92: 162, 1948.

# ASTUDY OF THE DEVELOPMENT OF TOLERANCE TO THE ACTIONS OF 1-METHYL-4-(3-HYDROXYPHENYL)-4-PIPERIDYL ETHYL KETONE HYDROCHLORIDE IN DOGS<sup>1</sup>

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Reports of the high analgesic activity and clinical use of 1-methyl-4-(3-hydroxyphenyl)-4-piperidyl ethyl ketone hydrochloride (Ketobemidone, WIN 1539) have appeared recently in the literature (1-7). We have reported in a previous publication (7) that tolerance developed to the analgesic action of WIN 1539 in rats at about the same rate as to that to morphine. In view of the importance of this factor in the treatment of chronic pain, it seemed advisable to determine the development of tolerance to the actions of WIN 1539 in another species.

**METHODS.** Dogs were selected as the experimental animals to facilitate the study of the actions of the compound coincidental with the analgesic effects in comparison with morphine. Six dogs were given WIN 1539 and three were given morphine sulfate. Aqueous solutions of the drugs were injected subcutaneously once daily. The initial dose of both drugs was 1.0 mgm./kgm. and as tolerance developed to the analgesic action the dosage was increased.

Analgesic action of the drugs was determined at weekly intervals by a modification of the Andrews and Workman method (8). The time, in seconds, necessary to elicit a muscle twitch was determined when a thermal stimulus of constant intensity was applied to a shaved, blackened area of the dog's back. The apparatus consisted of a portable light containing a shutter which was synchronized with an electric stop clock in such a manner that the shutter was opened and the clock started simultaneously. At the time the muscle twitch was noted the shutter was released which stopped the clock. The intensity of the stimulus was adjusted by means of a variable voltage transformer and standardized so that the normal reaction time was about 3.0 to 3.5 seconds. We observed that there was a large variation in reaction time obtained from different areas of the back. However, for any given small area the reaction time was constant. In order to minimize variation, the same area of the back was stimulated throughout each experiment. An increase in the time of exposure in analgesic testing greater than twice the normal exposure time will cause burning of the skin. For this reason, the maximum time of exposure was limited to 8 seconds and this time interval has been given the designation "complete analgesia". Even though no response could be elicited with the thermal stimulus in complete analgesia, a strong pinch of the skin would elicit a muscle twitch in some cases, indicating that the degree of analgesia was not complete to all methods of stimulation.

The hyperglycemic response was determined according to the method of Schales and Schales (7) on four dogs receiving WIN 1539 and two dogs receiving morphine. Blood sugar was determined on samples taken just prior to injection of the drug and at 1, 2, 3 and 5 hours following injection.

Other observations made at weekly intervals following the administration of the drugs

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<sup>1</sup> This work is part of an investigation carried out under the supervision of Dr. M. H. Seevers and submitted in a dissertation to the Graduate School of the University of Michigan in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

included heart rate, body temperature, respiration and other side-effects such as sedation, emesis, evidence of nausea, etc. Hematological examination was made at weekly intervals.

Administration of the drugs was discontinued at the end of seven weeks and the animals observed for possible abstinence symptoms. Test doses of the drugs were given at one and two week intervals after withdrawal in an effort to determine the degree of recovery from tolerance.

**RESULTS. 1. Analgesia.** The results obtained with WIN 1539 are given in table 1. The average analgesic response was lower after 22 daily doses of 1.0 mgm./kgm. than at the beginning of the drug administration, but this was due to the fact that only one animal gave a lower response. When the daily dose was increased to 5 mgm./kgm. for six days, a test dose of 1 mgm./kgm. produced a smaller

TABLE 1  
*Results of daily administration of WIN 1539*  
Figures are average values obtained from six dogs

DAY OF EXPERIMENT	DAILY DOSE	TEST DOSE	REACTION TIME		HEART RATE		RECTAL TEMPERATURE <sup>1</sup>		BLOOD SUGAR*		BODY WEIGHT
			Preinjection seconds	% Increase	Preinjection beats/min	% Decrease	Preinjection	% Decrease	Preinjection mgm/100 cc	% Increase	
	mgm./kgm.	mgm./kgm.									kgm.
1		1	35	100	105	30	102.7	3.9	108	31	13.2
8	1	1	33	100	104	30	102.3	2.7			12.8
15	1	1	33	100	100	10	102.3	4.1	106	19	12.8
22	1	1	31	94	96	26	102.0	3.9	111	21	12.9
29	5	1	29	67	108	46	102.2	4.4	114	0	13.2
36	5	5	32	100	119	57	102.1	4.6	113	28	13.2
43	10	5	27	100	95	46	102.3	4.6	111	40	12.8
49	20	5	35	89	130	60	102.6	5.4	117	13	12.3
Withdrawal											
7	..	1	32	90	106	38	102.2	2.9	...		13.6
14		1	34	100	97	36	101.2	2.3			13.8

\* Blood sugar values were obtained from four dogs

response than initially in all but one animal. There was no evidence of a decreased response to test doses of 5 mgm./kgm. until the daily dose had been increased to 20 mgm./kgm. After this dose had been given for five days the analgesic response to a 5 mgm./kgm. test dose was decreased in two animals but in the other four there was complete analgesia.

The development of tolerance to the analgesic action of morphine was more rapid than with WIN 1539. After fourteen days at a dose of 1 mgm./kgm. there was definite evidence of a decreased analgesic response but a dose of 5 mgm./kgm. on the fifteenth day produced complete analgesia. Tolerance developed to this dose in one week in two dogs and after two weeks in the other. As the dose was increased tolerance developed rapidly so that on the 49th day no analgesia was produced by doses of 40 mgm./kgm. in one dog and 80 mgm./kgm. in the other. One animal receiving morphine died on the 35th day of the experiment as a result of a technical accident.

Two weeks after withdrawal, all six dogs on WIN 1539 developed complete analgesia to a test dose of 1 mgm./kgm. The two dogs in the morphine group recovered from tolerance by the second week to the extent that complete analgesia was obtained with doses of 5 mgm./kgm. in one dog and 10 mgm./kgm. in the other.

2. *Heart Rate.* The average pre-injection heart rate of the WIN 1539 group was slightly greater at the end of drug administration than at the beginning. As the dose of the drug was increased the degree of bradycardia following drug injection became greater. This indicates no tolerance developed to this action of the drug. When the animals were tested with a 1.0 mgm./kgm. dose on the first and second week following withdrawal of the drug the pre-injection heart rate and the percentage decrease following the injection of the drug were essentially the same as at the beginning of the experiment.

The pre-injection heart rate of one dog receiving morphine had increased at the end of four weeks but it was no greater in the other two animals at the end of seven weeks of morphine administration than at the beginning. There was no significant change in the bradycardia which followed injection of the drug during daily administration.

3. *Body Temperature.* There was no appreciable change in the pre-injection rectal temperatures during the course of the experiment. The post-injection hypothermia produced by WIN 1539 increased slightly with the increase in dosage. We interpret this as an indication that no tolerance developed to this action of the drug.

There was an indication that tolerance developed to this action of morphine in one dog inasmuch as the decrease in body temperature became less as the daily administration was continued and the dose increased.

4. *Hyperglycemia.* There was no significant change in the average pre-injection blood sugar level during the course of the experiment. Tolerance developed to the hyperglycemic action of WIN 1539. A slight decrease in the hyperglycemia following the injection of the drug was observed after three weeks of daily administration of 1 mgm./kgm. When the dose was increased to 5 mgm./kgm./day for six days, a test dose of 1 mgm./kgm. on the following day caused no increase in blood sugar. There was evidence of tolerance to a test dose of 5 mgm./kgm. by the end of seven weeks.

One dog receiving morphine was found to have developed a tolerance to the hyperglycemic action of this drug by the third week. The other animal showed no hyperglycemic response to morphine at any time during the experiment. It may be assumed that this animal developed a tolerance during the administration of small doses since a hyperglycemia would be expected from the larger doses used here.

5. *Hematology.* There were no significant changes in hemoglobin concentration, hematocrit, red cell count or white cell count during the administration of these analgesic drugs or following their withdrawal.

6. *Respiration.* The effect of the analgesic drugs on respiration was not determined quantitatively because of the many factors which influence this action.

One consistent observation with both drugs, however, was an increase in respiratory rate which appeared five to fifteen minutes following injection of the drugs. This increase, which was so great that the dogs panted for several minutes, was followed by a marked decrease in rate which appeared to be associated with the degree of sedation produced by the drug. We observed no marked change in these responses during the time of daily drug administration.

*7. Other Side Effects.* Both drugs produced a marked sedation. However, it was not of such a degree as to be classed as narcosis. The animals were drowsy and quiet but could be aroused easily by a sharp noise. This action appeared within about 30 minutes after the injection of the drugs. The development of tolerance to this effect was evidenced by a decrease in the duration of action with continued daily administration of the drugs.

Vomiting occurred infrequently with morphine and was not observed in any of the dogs receiving WIN 1539.

A conditioned salivary response characterized by salivation preceding injection of the drugs appeared during the second week of the experiment. The flow of saliva increased after the injection of the drugs but usually ceased about an hour afterwards. Occasionally a slight rhinorrhea was observed to follow injection.

*8. Body weight.* Of the dogs receiving WIN 1539, three gained and the other three lost weight, whereas all of the dogs on morphine lost weight. The loss in weight was apparently due to a decreased intake of food inasmuch as the animals which lost weight refused to eat their regular rations. For this reason they were given supplemental feedings of milk and raw meat. Two of the dogs receiving morphine became constipated but there was no evidence of constipation in any of the animals receiving WIN 1539.

*9. Withdrawal Symptoms.* After the administration of analgesic drugs was discontinued, the animals were observed for abstinence symptoms. The average heart rate of the WIN 1539 group was only slightly higher 24 hours after withdrawal than on the day of withdrawal. The dogs receiving morphine had an increased heart rate which reached its maximum 48 hours following withdrawal. Twenty-four hours after withdrawal of WIN 1539 there was a slight increase in the average blood sugar level, but at 48 hours it was practically normal. We observed no pyrexia following withdrawal of the drugs. The most noticeable reaction seen was that of excitability. The animals appeared quite restless and whined and barked. They also evidenced a desire for attention. These reactions were most marked during the first day after withdrawal, and by the end of a week the animals appeared to be normal.

*Discussion.* From a practical standpoint, the most important consideration in the chronic administration of analgesic drugs is the development of tolerance to the pain-relieving action. Tolerance to other actions is variable both in degree and rate of development. Morphine tolerance in the dog has been studied by many investigators and there are a few reports describing the development of tolerance to some of the newer synthetic drugs. Scott *et al.* (10) observed that tolerance developed to the analgesic action of methadone and Wikler and

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Dr. E. W. McChesney, Dr. F. C. Goble, Mr. J. D. Frick and Miss B. L. Dertinger for their assistance in this work.

## REFERENCES

1. KLEIDERER, E. C., RICE, J. B., CONQUEST, V., AND WILLIAMS, J. H.: Report No. 931, Office of the Publication Board, Department of Commerce, Washington, D. C.
2. SCOTT, C. C., KOHLSTAEDT, K. G., AND CHEN, K. K.: *Current Res. Anesth. and Analg.*, 26: 12, 1947.
3. KIRCHHOF, A. C.: *Fed. Proc.*, 7: 234, 1948.
4. LUND, C. J.: *Am. J. Obst. and Gyn.*, 55: 1007, 1948.
5. CAHEN, R. L., ERSTEIN, H. J., AND KREMENTZ, C. S.: *THIS JOURNAL*, 94: 323, 1948.
6. MILLER, L. C.: *Ann. N. Y. Acad. Sci.*, 51: 34, 1948.
7. LEWIS, J. R.: *THIS JOURNAL*, 96: 31, 1949.
8. ANDREWS, H. L., AND WORKMAN, W.: *THIS JOURNAL*, 73: 99, 1941.
9. SCHALES, O., AND SCHALES, S. S.: *Arch. Biochem.*, 8: 285, 1945.
10. SCOTT, C. C., CHEN, K. K., KOHLSTAEDT, K. G., ROBBINS, E. B., AND ISRAEL, F. W.: *THIS JOURNAL*, 91: 147, 1947.
11. WIKLER, A., AND FRANK, K.: *Fed. Proc.*, 6: 384, 1947.
12. ISBELL, H., WIKLER, A., EDDY, N. B., WILSON, J. L., AND MORAN, C. F.: *J. A. M. A.*, 135: 888, 1947.
13. FINNEGAN, J. K., HAAG, H. B., LARSON, P. S., AND DREYFUSS, M. L.: *THIS JOURNAL*, 92: 269, 1948.



# THE PHARMACOLOGY OF THIOCYANOBENZOIC ACIDS

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Claude Bernard demonstrated the depressant action of the thiocyanate ion on the myocardium in 1857. Pauli (1) introduced the alkali thiocyanates into medicine as vasodilators in 1903. The use of simple routine methods to estimate the thiocyanate content of the blood was instrumental in creating renewed interest in thiocyanate therapy in hypertension (2). The success with thiocyanates in the treatment of hypertension has been the subject of much controversy. Their value in hypertension appears to be limited.

Taubman (3) studied pharmacologically a number of organic thiocyanates in 1930. Many aliphatic thiocyanates, such as methyl, ethyl and propyl, were studied on a small number of rabbits. Taubman observed that the aliphatic thiocyanates in general produced a pharmacologic syndrome consisting of convulsions, fall in body temperature and centric respiratory stimulation. Popov (4) showed that in general, aryl thiocyanates were less toxic than the alkyl compounds. Taubman studied para-thiocyananiline and observed blood changes in animals similar to those produced by aniline.

Through the kindness of Dr. W. H. Hill<sup>1</sup> a large number of aryl thiocyanates were made available to us for study. Among these compounds, para-thiocyanophenol appeared to be the most active on the circulation. Owing to the irritating properties of the compound on the skin and mucous membranes, and the inherent toxicity of phenol, we suggested the synthesis of the corresponding benzoic acid derivative. Accordingly, meta- and para-thiocyanobenzoic acids were synthesized. Each is a grayish-white powder, relatively insoluble in water, but easily made soluble by the addition of a mole of sodium bicarbonate. Our experimental studies were conducted with the sodium salts of these two isomeric thiocyanobenzoic acids.

*Blood pressure studies—dogs and rabbits.* The blood pressure studies were conducted on animals under ether anesthesia. Carotid artery blood pressure was recorded and injections of the various compounds were made into the saphenous vein. One per cent solutions of the sodium salts of the meta- and para-thiocyanobenzoic acids were injected on the basis of 0.5 to 1 cc./kgm. Eight dogs and four rabbits were used in the study of the meta compound and ten dogs were employed in the study of the para compound. The injections caused a prompt fall in blood pressure which extended approximately fifteen minutes. The potencies of the two isomers were found to be the same.

A typical response compared with the action of sodium thiocyanate is shown in figure 1.

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An examination of the figure reveals the depressor potency of the aryl thiocyanate compared with the thiocyanate ion. In many comparative studies on dogs, it appears that the thiocyanate ion is approximately  $\frac{1}{75}$  as potent as the thiocyanate radical in thiocyanobenzoic acid when calculated on a molar basis. An examination of the tracing in figure 1 shows that the depressor response elicited by thiocyanobenzoic acid is accompanied by an increase in the amplitude of respiration.

At the peak of the effect produced by thiocyanobenzoic acid, the blood ionic thiocyanate level was not increased. It appears, therefore, that this depressor response is elicited by the intact molecule. The depressor action was not affected

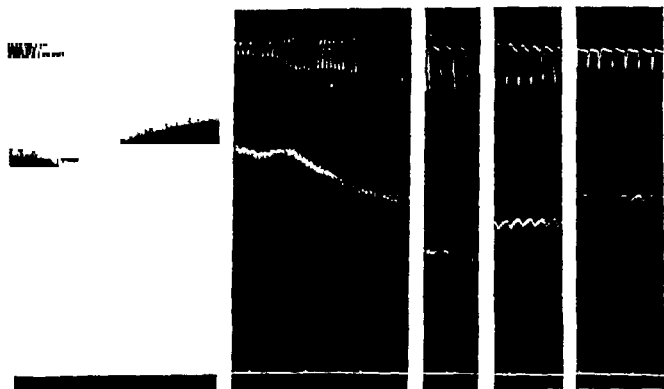


FIG 1 Effect of *m*-Thiocyanobenzoic Acid and Sodium Thiocyanate on the Blood Pressure of the Dog (Female, 6.6 kgm ether anesthesia, intravenous injections, normal blood pressure 172 mm Hg)

a 2 cc/kgm 2 per cent solution of sodium thiocyanate b 0.5 cc/kgm 0.5 per cent solution of sodium *m*-thiocyanobenzoate c After 4 minutes d After 16 minutes e After 23 minutes

by the atropinization of the animal. Neither tetraethylammonium chloride (Etamon) nor dibenzyl- $\beta$ -chloroethylamine (Dibenamine) obliterated the fall in blood pressure produced by thiocyanobenzoic acid. In four dogs, thiocyanobenzoic acid elicited its typical response after the destruction of the brain. Therefore the depressor response produced by thiocyanobenzoic acid is not neurogenic in character. The locus of action was sought on the vascular smooth musculature or the heart.

Electrocardiographic studies on six dogs before and after the injection of thiocyanobenzoic acid revealed that the compound produced changes in the rate, form and regularity of the electrocardiogram. The most constant finding was a marked accentuation of the T-wave. This is illustrated in figure 2.

It is of interest that the marked accentuation of the T-wave was also the most

prominent feature of the electrocardiogram of the dogs fed the compound repeatedly (200 mgm. daily) over a period of 30 days.

*Perfused leg vessels of the frog.* The effect of thiocyanobenzoic acid upon the perfused leg vessels of the frog was studied in ten frogs by the Trendelenberg technic. Perfusion was conducted with a 1:1000 concentration of the sodium salt of para-thiocyanobenzoic acid in Howell-Ringer's solution. Twenty measurements of

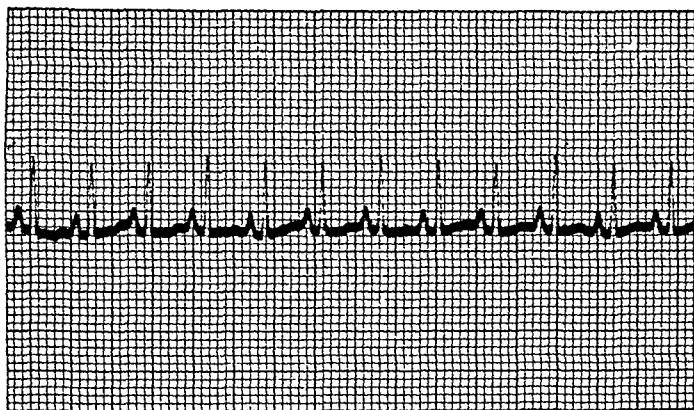


FIG. 2A

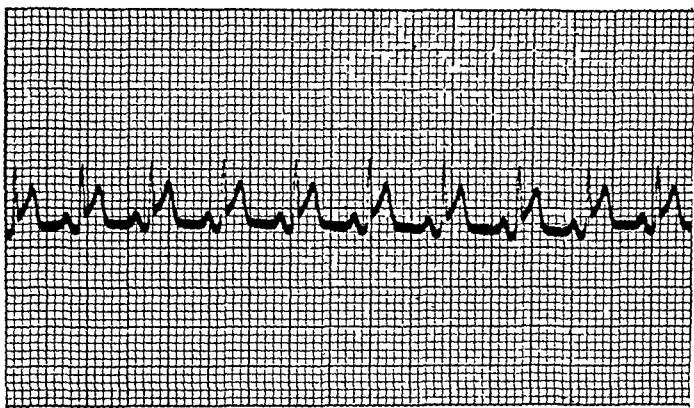


FIG 2B

FIG. 2. Electrocardiograms of Dog, Lead II.

a. Before injection. b. Immediately after thiocyanobenzoic acid.

rate of flow were made on the ten frogs. There was invariably evidence of dilatation; the mean increase was 11.3 per cent. This action was not shared by the thiocyanate ion.

*Action on smooth muscle.* The action of the thiocyanobenzoic acids was studied in six experiments on the smooth musculature of the rabbit's intestine and the rat's uterus *in vitro*. The addition of 2 mgm. of para-thiocyanobenzoic acid to 30 cc. of the bath depressed promptly the frequency and amplitude of contractions. This action was not shared by the thiocyanate ion, as shown in figure 3.

*Action on cardiac output of frog* In eight experiments of cardiac perfusion of the frog's heart *in situ* (Sollman and Bulow, 1926) para thiocyanobenzoic acid was found to reduce the cardiac rate and amplitude of contraction. The average decrease in cardiac output produced by perfusing a 1:500 solution of the thiocyanate compound was 33 per cent.

*Acute Toxicity (White Rat)* Acute toxicity determinations were carried out on the white rat by intraperitoneal injection and oral administration. These data were compared with the toxicity of sodium thiocyanate. Fatal doses of the meta- and para-thiocyanobenzoic acids produced hypermilitability, hyperreflexia, tremors and short convulsive seizures. This syndrome was followed rapidly by

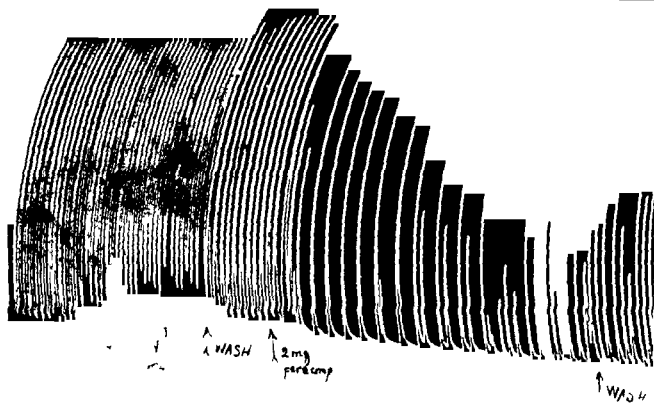


FIG. 3. Effect of p-Thiocyanobenzoic Acid on the Isolated Rat's Uterus

weakness of the legs and extreme flaccidity. The animals died of respiratory failure. The heart continued to beat for some time after the cessation of respiration. Fatal doses killed within 30 minutes. One hundred and eight animals were used and the  $LD_{50}$  was determined by Trevan's method.

For meta-thiocyanobenzoic acid by intraperitoneal injection the  $LD_{50}$  was found to be 17 mgm/kgm,  $SE = \pm 1.3$  mgm. For the para compound the  $LD_{50}$  was 22 mgm/kgm,  $SE = \pm 1.9$  mgm. For sodium thiocyanate the  $LD_{50}$  was found to be 510 mgm/kgm,  $SE = \pm 42.5$  mgm. By oral administration the  $LD_{50}$  for the para compound was 83 mgm/kgm,  $SE = \pm 8.1$  mgm, and for the meta derivative 73 mgm/kgm,  $SE = \pm 5.7$  mgm.

*Effect on cytochrome oxidase and reductase* A suspension of rat's brain brew supplied the cytochromes for these experiments. Quantities of 2 mgm per 5 cc of reaction mixture of either meta- or para-thiocyanobenzoic acid inactivated

cytochrome oxidase, as shown by the Nadi reaction. Neither 2 nor 5 mgm. of sodium thiocyanate interfered with the oxidase activity. Five experiments were conducted.

Cytochrome reductase action was determined by the Thunberg method. Quantities of 1 mgm. per cc. of reaction mixture of either of the isomeric benzoic acids prevented the decolorization of methylene blue solution. The same quantity of sodium thiocyanate produced no significant effect. Ten experiments were conducted.

*Fate in the body.* It was considered of interest to determine whether or not body tissues were capable of converting these aryl thiocyanates into thiocyanate ions. The undecomposed molecules of the thiocyanobenzoic acids do not react with ferric salts to produce the characteristic color of ferric thiocyanate. Hence the usual clinical methods for the determination of the thiocyanate ion content of blood were applicable to these studies.

Fifteen rats and four dogs were given oral doses of either para- or meta-thiocyanobenzoic acid on a basis of 20 mgm./kgm. The thiocyanate ion made its appearance slowly in the blood. Concentrations of 2 mgm. per cent occurred in each species in approximately six hours. A peak of 4 mgm. per cent was reached in ten to fifteen hours, and the diminution of this blood value proceeded slowly for several days.

It is apparent that tissues are capable of hydrolyzing the compounds into thiocyanate ions and benzoic acid. It is assumed that the latter compound follows its usual course of excretion.

*Chronic feeding studies.* Two dogs, 6 to 8 kgm., were given 200 mgm. of para-thiocyanobenzoic acid daily by mouth for a period of 30 days. The compound stimulated prompt bowel evacuation at first, after which tolerance was acquired. The animals maintained their body weights and showed no visceral damage at the end of the feeding period. Electrocardiograms taken at the end of the feeding period revealed a strongly accentuated T-wave. At the end of this period, the animals were sacrificed and the viscera were examined histologically. They were all normal except the thyroid gland, which showed hyperplasia.

**DISCUSSION.** The thiocyanate radical attached to the phenyl group as it appears in the thiocyanobenzoic acids studied is a potent depressor agent. The thiocyanate radical, as present in this compound, upon intravenous injection in the dog is approximately 70 times more potent than the thiocyanate ion. The action is apparently due to the intact molecule and is produced by cardiac slowing and peripheral vasodilatation. The toxicity of the thiocyanobenzoic acids is far greater than that of the alkali thiocyanates. These organic thiocyanates interfere with the activity of cytochrome oxidase and reductase, which action is not shared by like concentrations of sodium thiocyanate.

#### SUMMARY

Meta- and para-thiocyanobenzoic acids have been studied and compared with the action of alkali thiocyanates. Depressor potency is greatly enhanced by

the conversion of ionic thiocyanate into an aryl thiocyanate substitution compound.

## REFERENCES

1. PAULI, W.: *Münch. Med. Wochenschr.*, 50: 153, 1903.
2. BARKER, M. H.: *J. A. M. A.*, 106: 762, 1936.
3. TAUBMANN, G.: *Arch. f. exper. Path. u. Pharmacol.*, 160: 57, 1930.
4. POPOV, P. V., AND ILLINKA, M. I.: *Trans. Sci. Int. Fertilizer's Insectofungicides*, 135: 156, 1939.

## STUDIES ON VERATRUM ALKALOIDS

### VIII. VERATRAMINE, AN ANTAGONIST TO THE CARDIOACCELERATOR ACTION OF EPINEPHRINE

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While several groups of compounds are known which are able to annul the vasopressor action of epinephrine, no substance has hitherto been found capable of inhibiting its cardioaccelerator action. Among the veratrum alkaloids there are pure, crystalline compounds which possess the ability to prevent or abolish the cardioaccelerator (or positive chronotropic) action caused by epinephrine. The following is a description of the phenomenon as observed with veratramine.

Veratramine,  $C_{27}H_{39}O_2N$ , was isolated by Saito (1) from the Japanese *Veratrum grandiflorum* Loes fil.; it was found by Jacobs and Craig (2) in the American *Veratrum viride*, Aiton. Professor Walter A. Jacobs of the Rockefeller Institute of Medical Research made the veratramine available for the present pharmacological investigation.

**METHODS.** Experiments on the isolated heart were conducted in 22 heart-lung preparations of the dog (HLP). The method used was described elsewhere in detail (3, 4). The dogs weighed between 7 and 14.1 kgm. Total blood volume was between 500 and 900 cc. at the beginning of the experiments. Cardioacceleration was produced by epinephrine injection in single doses in a few cases; in most cases, continuous infusions were made with an automatically driven syringe (5) delivering between 1 and 100 microgm. of epinephrine per minute. Epinephrine bitartrate<sup>1</sup> was administered, as a rule in concentrations of 1:5000 or 1:10000 (except in a few cases in which 1:100000 was used) in 0.9 per cent sodium chloride solution. All doses refer to the synthetic *l*-epinephrine base.

Experiments with the circulation intact were conducted in four dogs, weighing between 8.5 and 14 kgm., under pentobarbital (35 mgm. per kgm.) or Dial-urethane, containing per cc. 0.1 gm. diallylbarbituric acid, 0.4 gm. urethane, and 0.4 gm. monoethylurea in water (0.7 cc. per kgm.) anesthesia; and in eight cats, weighing between 1.7 and 3.0 kgm. Two of the cats were spinal and three were completely pithed preparations. Decerebration and destruction of the medulla were carried out by entering the fourth ventricle through the atlanto-occipital membrane. In the intact cats pentobarbital or Dial-urethane was used as general anesthetic. Blood pressure was recorded from the carotid or femoral artery with a mercury manometer. Epinephrine was given by continuous infusion into the external jugular vein; concentrations were, as a rule, 1:2000 or 1:5000 in 0.9 per cent sodium chloride solution.

Heart rates in all experiments were counted from permanent electrocardiographic records obtained with a Grass ink-writing oscillograph.

The sample of veratramine used had a rotation of  $[\alpha]_D^{27} = -69.0^\circ$  ( $c = 0.98$  in methanol). It melted at 204–207° C. after preliminary sintering (2). The veratramine base was brought into solution with 0.2 cc. of *N*/10 hydrochloric acid for every 5 mgm. of the alkaloid and made up to a concentration of 1:1000 with distilled water. Such solutions (pH 4–5) were kept at room temperature (approximately 25° C.) for up to one month and showed no decrease or change in activity. All veratramine doses refer to the base.

<sup>1</sup> Epinephrine bitartrate generously supplied by Winthrop Chemical Company, Inc.

Atropine was administered in the form of the sulfate in a concentration of 1 per cent in 0.9 per cent sodium chloride solution. All doses refer to atropine sulfate.

**Results. I. The cardioaccelerator action of epinephrine in the isolated heart.** When epinephrine is administered continuously at a rate of 3-10 microgm. per minute in the HLP of the dog, the heart rate increases from the normal rate of the denervated heart to a steady level, its height depending upon the rate of administration (figure 1, see also figures 4 and 5). The level is not reached abruptly but requires ten to twenty minutes or more. Upon discontinuation of infusion the heart rate decreases first steeply, then more gradually, until the normal pre-infusion rate is again reached. Figures 1, 4 and 8 show this process to require a considerable time, approximately 50-60 minutes.

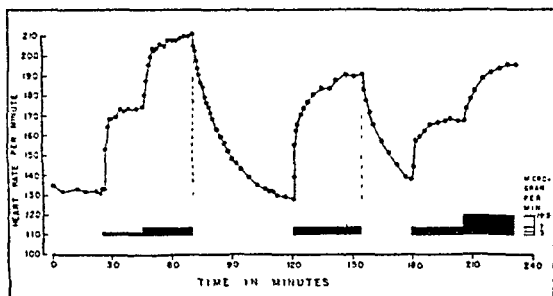


FIGURE 1. THE POSITIVE CHRONOTROPIC EFFECT OF EPINEPHRINE ADMINISTERED INTERMITTENTLY BY CONTINUOUS INFUSION

Dog, female, 14.1 kgm. Heart-lung preparation. The total blood volume was 600 cc. at the beginning. The mean arterial pressure was 112 mm.Hg. The systemic output was 500 cc. The temperature varied between 38.5-39.5° C. Black bars: continuous infusion of epinephrine (1:5000); calibration at right in microgm. of base per minute.

Upon repetition it is difficult with the same rate of infusion to reach the same level of elevation of heart rate (see figure 1) and considerably higher rates of infusion are needed to achieve this. When infusion of epinephrine is carried out without interruption for long periods of time, heart rate as a rule decreases little over a period of three hours (figure 2). In one experiment on the heart of an old dog with obvious signs of previous cardiac disease, the decrease proceeded at a faster rate. The causes for the difference in the response of isolated hearts to the chronotropic effect of such intermittent or uninterrupted continuous infusions of epinephrine are not at present known.

Unless effects of a drug, antagonizing cardioacceleration by epinephrine, are abrupt, caution is necessary in interpreting the results. It is safe to rely on the results obtained if the drug is given at the height of the epinephrine effect and acts abruptly. When the drug is administered after recovery from one or more infusion periods, differences in the response to intermittent epinephrine infusion



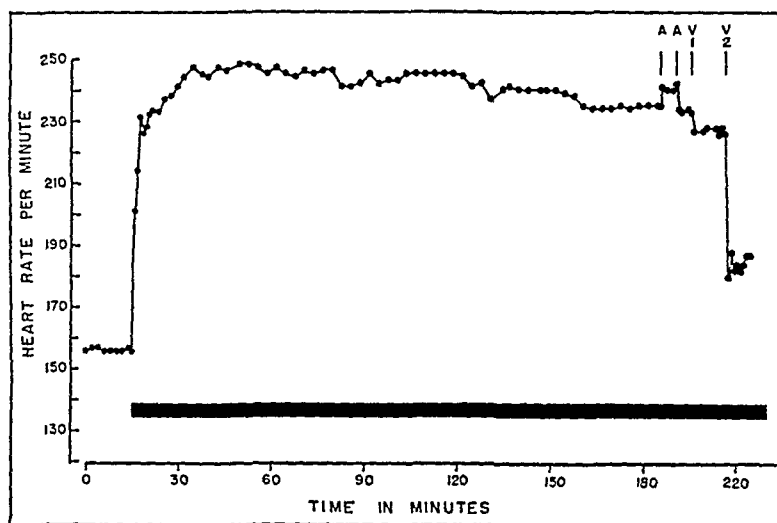


FIGURE 2. PERSISTENCE OF POSITIVE CHRONOTROPIC EFFECT OF LONGLASTING CONTINUOUS INFUSION OF EPINEPHRINE

Dog, male, 11.6 kgm. Heart-lung preparation. The total blood volume was 800 cc. at the beginning. The mean arterial pressure was 110 mm.Hg. The systemic output was 450 cc. The temperature varied between 38.2–39.2° C. Black bar: continuous infusion of epinephrine (1:5000) at the rate of 7 microgm. per minute. At A: 5 mgm. atropine sulfate injected. At V<sub>1</sub>: 0.1 mgm., at V<sub>2</sub>: 0.3 mgm. veratramine injected.

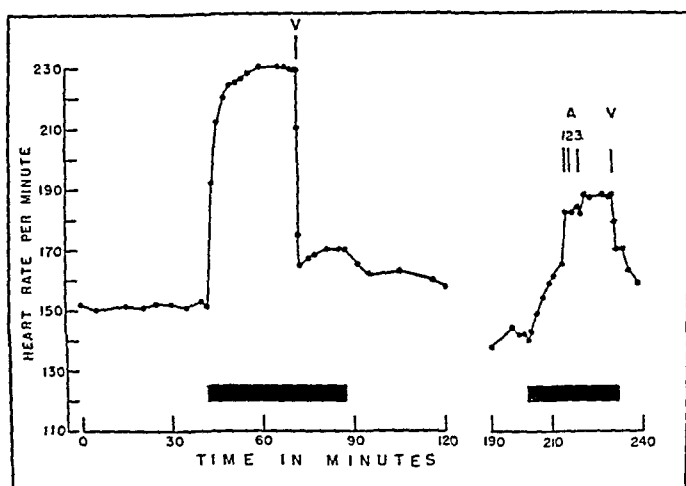


FIGURE 3. THE INHIBITION OF THE CARDIOACCELERATOR ACTION OF EPINEPHRINE BY VERATRAMINE

Dog, male, 9.3 kgm. Heart-lung preparation. The total blood volume was 800 cc. at the beginning. The mean arterial pressure was 106 mm.Hg. The systemic output was 400 cc. The temperature varied between 38.5–39.2° C. Black bars: continuous infusion of epinephrine (1:100000) 6.5 microgm./minute. At V: 0.5 mgm. veratramine was injected. At 1, 2, 3: 1, 3, and 10 mgm. atropine sulfate, respectively, were injected.

are less reliable as indicators of the inhibitory action on the cardioacceleration caused by epinephrine.

II. *Effect of veratramine in the isolated heart. a. Continuous infusion of epinephrine.* When veratramine is given to a HLP with an elevated heart rate due to continuous infusion of epinephrine, a decrease in rate occurs when doses of the order of 0.1 to 1.0 mgm. are injected. With this dosage no irregularities are observed and the rhythm continues to be a regular sinus rhythm. If the heart rate is elevated 50 to 80 per cent or more (requiring infusion of 5 to 10 microgm. of epinephrine per minute) 0.1 mgm. shows a noticeable, 0.3 mgm., a marked effect (see figure 2); 0.5 mgm. reduces the acceleration by 80 per cent or more and 1

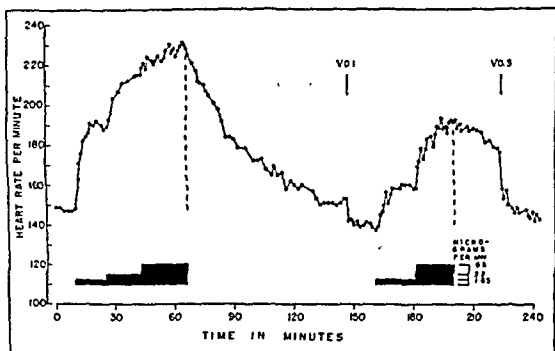


FIGURE 4. THE EFFECT OF VERATRUMINE ON THE POSITIVE CHRONOTROPIC ACTION OF EPINEPHRINE ADMINISTERED BY INTERMITTENT CONTINUOUS INFUSION

Dog, female, 13.2 kgm. Heart-lung preparation. The total blood volume was 800 cc. at the beginning. The mean arterial pressure was 112 mm.Hg. The systemic output was 450 cc. The temperature varied between 36.5-37.8° C. Black bars: continuous infusion of epinephrine (1:100000); calibration on right in microgm. per min. The vertical broken lines indicate the end of continuous infusion. At V: 0.1 and 0.3 mgm. of veratramine, respectively, were given.

mgm. causes a decrease in rate to normal or below. The decrease in rate is abrupt. The maximal effect is attained within one to three minutes and afterwards the heart rate tends to stabilize at a somewhat higher level. If epinephrine infusion is discontinued, heart rate, as a rule, gradually decreases to a lower level (figure 3).

In figure 4 the response of a heart to epinephrine infusion before and after 0.1 mgm. veratramine appears to indicate a marked effect of this dose of the alkaloid. The difficulty of reliably evaluating such a result was mentioned above; on the other hand, the abrupt break caused by 0.3 mgm. veratramine in the curve of decreasing heart rate leaves no doubt of the characteristic activity of the compound.

In the HLP, the antagonistic effect of veratramine to epinephrine acceleration is long lasting, irrespective of whether the epinephrine infusion is interrupted or continues for several hours. No recovery could be seen within two hours after 1 mgm. veratramine. Repeating the same epinephrine infusion in the experiment of figure 3 after two hours increased the heart rate from 142 to 165 (16 per cent), while during the preliminary test, the increase was from 150 to 230 (53 per cent).

If, after the veratramine has taken effect, the blood of the HLP is replaced repeatedly by fresh blood, the normal responsiveness of the heart to the chrono-

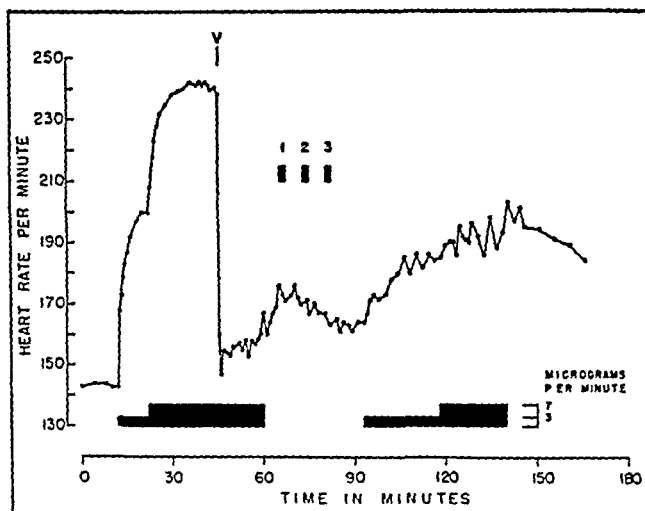


FIGURE 5. THE INFLUENCE OF BLOOD REPLACEMENTS ON THE INHIBITION BY VERATRAMINE OF EPINEPHRINE CARDIOACCELERATION

Dog, female, 9.25 kgm. Heart-lung preparation. The total blood volume was 400 cc. at the beginning. The mean arterial pressure was 112 mm. Hg. The systemic output was 400 cc. The temperature varied between 39.2-39.9°C. Black bars: continuous infusion of epinephrine (1:5000); calibration on right in microgm. of epinephrine per minute. At V: 0.5 mgm. veratramine was injected. At 1, 2 and 3 the major part of blood was removed and blood containing no veratramine was substituted (350 cc. of fresh blood at 1 and 2; 175 cc. of fresh blood at 3).

tropic property of epinephrine is not restored (figure 5). The effect of veratramine cannot be washed away.

Although markedly reduced by veratramine, the responsiveness of the heart to the accelerator effect of epinephrine is not entirely abolished by this compound. While a certain infusion rate of epinephrine may no longer increase heart rate, a sufficiently high concentration of epinephrine reaching the heart still has qualitatively its characteristic positive chronotropic effect. The rapid injection of large single doses of epinephrine (or norepinephrine), readily overcomes the reduced sensitivity of the heart and leads to very marked increases in rate (see figure 8).

b. *Modification of the chronotropic effect of single doses of epinephrine by veratramine.* For the reason mentioned in the last paragraph it is not quite as easy

as with continuous infusion of epinephrine to show the striking antagonistic effect of veratramine when single injections of epinephrine are used, especially if, as is customary, these are given within a few seconds. With this technique the epinephrine concentrations reaching the heart are initially quite high and therefore relatively large doses of veratramine are necessary to give clear results. After the response of the heart to 10 microgm. of epinephrine had been established in the experiment of figure 6 (see curves 1 and 2), 3 mgm. of veratramine caused a decrease in rate from 128 to 73 per minute. The heart continued beating at a

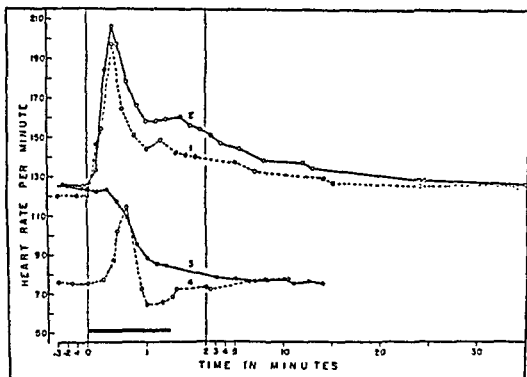


FIGURE 6. THE EFFECT OF VERATRUMINE ON THE POSITIVE CHRONOTROPIC ACTION OF SINGLE DOSES OF EPINEPHRINE

Dog, male, 12 kgm. Heart-lung preparation. The total blood volume was 800 cc. at the beginning. The mean arterial pressure was 100 mm. Hg. The systemic output was 400 cc. The temperature varied between 38.4-38.8° C. All injections were started at zero time. Curves 1, 2 and 4 were obtained with 10 microgm. epinephrine given within three seconds. Curve 3 represents the heart rate change caused by 3 mgm. veratramine given over a period of 83 seconds (length of black bar). The last point of curve 1 is identical with the first point of curve 2. Between the last point of curve 2 and the first point of curve 3 five minutes elapsed. The last point of curve 3 is identical with the first point of curve 4.

Note the change in time scale between the two vertical lines during minutes 1 and 2.

regular sinus rate (curve 3). After this, the chronotropic effect of 10 microgm. of epinephrine was markedly changed (curve 4). The maximum reached was only 115 beats per minute, but this still represented a relative increase of 57 per cent, as compared to approximately 60 per cent in the controls. There was a delay of about ten seconds in the onset of cardioacceleration. The maximum of the rate likewise was delayed. The rate returned abruptly to the pre-injection level or slightly below. The total time of rate increase was compressed into a period of 30 seconds, instead of taking 20 to 30 minutes as after the control injections.

If single injections are made during one minute to prevent excessively high peak concentrations from reaching the heart, as little as 0.1 mgm. veratramine

also shows the reduction of sensitivity of the heart to the cardioaccelerator action of epinephrine. In figure 7 administration of 10 microgm. of epinephrine (prior to 0.1 mgm. veratramine) increased the heart rate from 133 to 169 (27 per cent). Thirty minutes after veratramine the same dose increased the rate from 125 to 140 (12 per cent). Duration of action likewise was reduced. Subsequently the effect continued undiminished and one and one-half hours after veratramine, 10 microgm. of epinephrine increased the rate from 123 to 133 (8 per cent). Even a dose of 100 microgm. was now no more active than the dose of 10 microgm. prior to the administration of veratramine.

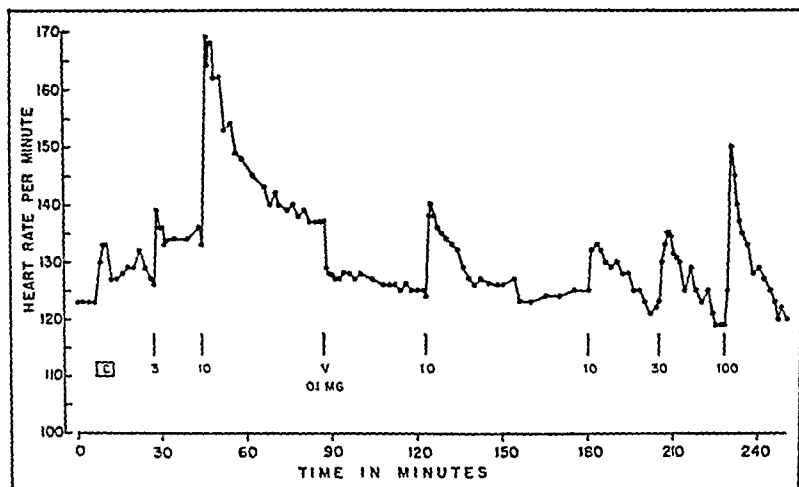


FIGURE 7. THE EFFECT OF VERATRAMINE ON THE POSITIVE CHRONOTROPIC ACTION OF SINGLE DOSES OF EPINEPHRINE

Dog, male, 11.6 kgm. Heart-lung preparation. The total blood volume was 800 cc. at the beginning. The mean arterial pressure was 115 mm.Hg. The systemic output was approximately 470 cc. The temperature varied between 38.4–39° C. The figures below the signals mean microgm. of epinephrine injected in 60 seconds into the blood returning to the venous supply reservoir. At C: a competence test was conducted. At V: 0.1 mgm. veratramine was injected.

c. *The effect of atropine.* In his "law of the antagonism of the cardiac nerves", Hunt (6) established the fact that submaximal stimulation of accelerator and decelerator nerves of the heart, made simultaneously, antagonize each other so that the result is the arithmetical mean of the pure accelerator and the pure decelerator effects. This implies that no change in rate may occur with appropriate intensities of submaximal stimulation. There is the possibility that veratramine might stimulate the peripheral parasympathetic neuron in the heart. To test whether such a mechanism mediated by acetylcholine and sensitive to atropine might play a rôle in the phenomenon observed with veratramine, atropine was administered to the HLP in doses of between 5 and 20 mgm. to obviate such effects.

In figure 8, after establishing the response of the heart to epinephrine infusion

and allowing normal rate to return, veratramine was given in two doses of 0.1 and 0.3 mgm. Atropine sulfate in a dose of 10 mgm. did not change the rate markedly within five minutes and did not prevent the subsequent dose of 1 mgm. veratramine from decreasing heart rate, nor did it restore the ability of the heart to respond to the epinephrine infusion started three minutes after the last veratramine dose. An additional dose of 10 mgm. atropine sulfate also did not modify the heart rate response to the epinephrine infusion within the following period of fourteen minutes. A very marked, though short lasting, positive chronotropic effect could still be elicited when 1 mgm. of *dl*-norepinephrine was given.

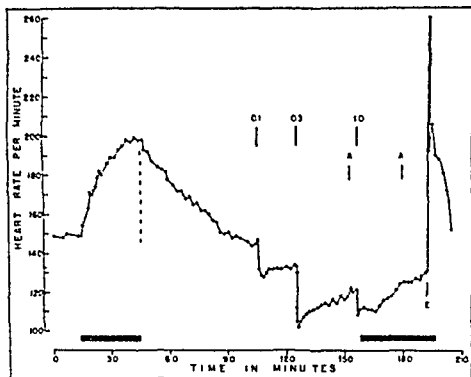


FIGURE 8 THE INHIBITION OF EPINEPHRINE CARADIOACCELERATION BY VERATRAME IN THE ISOLATED HEART UNDER ATROPINE

Dog, male, 11.7 kgm. Heart-lung preparation. The total blood volume was 800 cc at the beginning. The mean arterial pressure was 112 mm Hg. The systemic output was approximately 550 cc. The temperature varied between 36-36.7° C. Black bars: continuous infusion of epinephrine (1:100000) 1.65 microgm per minute. At respective signs: 0.1, 0.3 and 1.0 mgm veratramine. At A: 10 mgm of atropine sulfate. At D: 1 mgm of *dl*-norepinephrine, while continuous infusion of epinephrine was going on. The vertical broken line indicates the end of the epinephrine infusion.

While the first dose of atropine frequently does increase heart rate after veratramine (see figure 3), this increase always is of the order observed in the normal HLP. Figure 2 proves that atropine, given during the epinephrine infusion, is unable to prevent the antagonistic action of veratramine to the cardioaccelerator effect of epinephrine in the isolated heart.

III. *The effect of veratramine on the cardioaccelerator action of epinephrine upon the heart in situ.* a. *Dose, intensity and duration of the veratramine effect.* In the circulatory system of the anesthetized dog and cat and of the spinal or completely pithed cat the positive chronotropic effect due to continuous infusion of epinephrine differs from that in the HLP in that the attainment of a steady level

proceeds faster and the rate of disappearance of the epinephrine effect also is greater than in the isolated heart. Furthermore, under these conditions intermittent infusion of doses of epinephrine which cause increases in rate to between 250 and 280 beats per minute does not appear to lead to the marked decline of responsiveness observed in the HLP (see figure 1).

The antagonistic effect of veratramine to epinephrine cardioacceleration *in situ*, under the stated conditions, occurs as abruptly as in the isolated heart (figure 9), and peak effect is likewise reached within one to three minutes. The abruptness of the effect is evident from a comparison of the action of 85 microgm. per kgm.

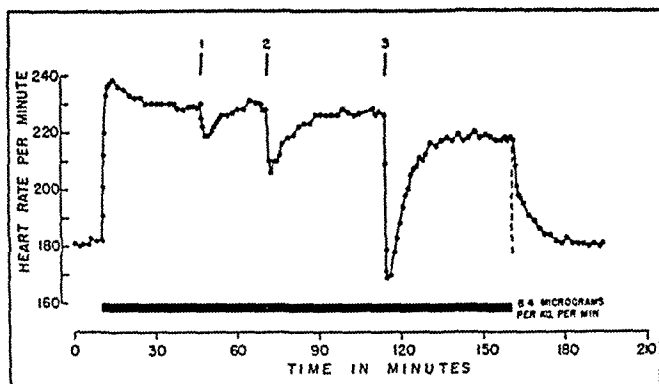


FIGURE 9. THE INHIBITORY EFFECT OF GRADED DOSES OF VERATRAMINE ON THE CARDIOACCELERATION CAUSED BY EPINEPHRINE

Dog, male, 11.7 kgm. Nembutal anesthesia. Artificial respiration, open chest, vagi crushed in neck. Anesthesia was maintained during experiment by constant i.v. drip of 10-15 drops of a 0.07 per cent sodium pentobarbital solution per minute. The blood pressure was recorded from the left femoral artery. At the beginning, before the epinephrine infusion began, it was 106 mm.Hg. At the end of the experiment, after the epinephrine infusion had been stopped, it was 78 mm.Hg. The rectal temperature varied between 38.4 and 38.9° C. Black bar: continuous infusion of epinephrine (1:1000) at the rate of 8.4 microgm. per kgm. per minute. At 1, 2, 3: 8.5, 25.5 and 85 microgm. of veratramine per kgm., respectively, were injected i.v. The vertical broken line indicates the end of the continuous infusion of epinephrine.

(figure 9, 3) with the curve of heart rate decrease after the subsequent discontinuation of epinephrine infusion.

Contrary to the observations in the HLP, the veratramine effect wears off in the whole animal. With the smallest effective dose of 8.5 microgm. per kgm. (figure 9, 1) it had disappeared within seventeen minutes. As the dose increased, intensity and duration of action correspondingly increased and the heart rate did not completely return to the pre-injection level but remained at a lower plateau (see figures 9 and 10). An analysis will be presented elsewhere of the rôles which distribution and elimination of veratramine play in the disappearance of its effect.

Upon discontinuation of the continuous infusion of epinephrine, heart rate drops to a lower level. With doses of veratramine leading to a persistent level of heart rate little below the maximal elevation caused by epinephrine, the rate of

deceleration is not different from that after ordinary injection or infusion of epinephrine, and the heart rate does not fall below the normal level (figure 9).

In the experiment of figure 10 in which the second veratramine dose brought the heart rate to the normal level, the rate dropped below normal upon discontinuation of epinephrine infusion. Renewed continuous infusion of the same amount of epinephrine restored the previous level, indicating that maintenance of this level was the resultant of the simultaneous action of the veratramine

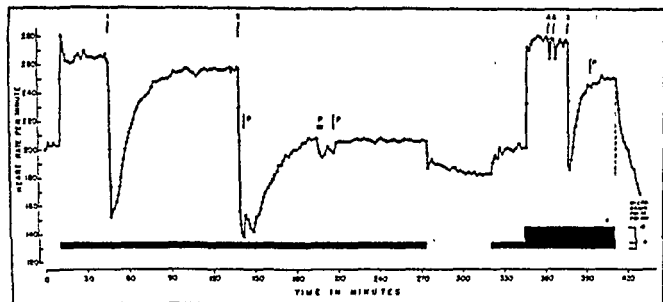


FIGURE 10. THE EFFECT OF VERATRUMINE ON THE CARDIOACCELERATION CAUSED BY CONTINUOUS INFUSION OF EPINEPHRINE

Dog, female, 6.85 kgm. Sodium pentobarbital anesthesia. Anesthesia was maintained by a constant intravenous drip of 10-15 drops per minute of a 0.07 per cent sodium pentobarbital solution throughout the experiment. The rectal temperature of the animal varied between 39 and 40° C. At the beginning, before the epinephrine infusion, the mean arterial pressure was 125. mm.Hg. At the end of experiment (7 hours later) during the epinephrine infusion, it was 99 mm.Hg. After epinephrine was discontinued, it dropped to 40 mm.Hg. Vagi severed in the neck. Two doses of 5 mgm. atropine sulfate each were given prior to the start of the experiment. The black bars indicate continuous infusion of epinephrine (1:2000) into the right external jugular vein; calibration on right in microgm. of base. Single intravenous injections were made into right femoral vein. At 1 and 3: 0.1 mgm. veratramine per kgm. intravenously. At 2: 0.3 mgm. veratramine per kgm. intravenously. At P: 35 mgm. sodium pentobarbital injected intravenously in a single dose. At second P: 35 mgm. were given in four fractional doses. At A 5 mgm. atropine sulfate injected intravenously. The broken vertical line near the end indicates the end of the continuous infusion of epinephrine.

present and of the concentration of epinephrine maintained in the pacemaker tissue by the epinephrine infusion. In order to reach the initial elevation of rate, originally achieved by the infusion of 4 microgm. of epinephrine per kgm. per minute, four times the amount of epinephrine (15 microgm./kgm./min.) was now required. In spite of the high infusion rate of epinephrine, a further dose of veratramine (figure 10, 3), equivalent to the first dose (figure 10, 1) still showed the characteristic effect although, quantitatively, peak action was less pronounced in intensity, and the time required to reach a steady level was shorter in the presence of the higher concentration of epinephrine.



b. *The effect of atropine.* As in the HLP, atropine does not prevent or abolish the effect of veratramine on the heart *in situ*. This has been shown not only in animals under barbiturate anesthesia (figure 10, 3) but also in spinal animals. In the experiment of figure 11 A and B the three successive administrations of 0.2 mgm. veratramine per kgm. resulted in increasing intensity of peak action although the rate of infusion of epinephrine was increased after the first dose. Atropine did not modify the veratramine response even if given in a dose of 3.3 mgm. per kgm. immediately preceding the injection of veratramine (see figure 11 B; A<sub>2</sub>).

c. *Action of veratramine upon the central nervous system and the effect of general anesthesia on the veratramine effect.* The only side effect of veratramine observed with the dosage range employed in these experiments was an action upon the central nervous system. In the intact animal without anesthesia signs of hyperexcitability begin to occur with doses between 0.1 and 0.2 mgm./kgm. and convulsions occur with doses between 0.2 and 0.3 mgm./kgm. The convulsions are of a clonic nature.

In the experiment of figure 10 with the animal under anesthesia no signs of central nervous system stimulation were noticeable until shortly after the injection of 0.3 mgm. veratramine per kgm. at 2. A dose of 35 mgm. sodium pentobarbital (at time 140) abolished the convulsions temporarily. They reappeared 30 minutes later and became marked; additional doses of sodium pentobarbital (between time 200 and 210) restored complete anesthesia. The animal remained quiet and there was no sign of hyperexcitability when tendon reflexes or the lid reflex were tested, even after heart rate had been restored to the elevated level of 260 per minute by the continuous infusion of 15 microgm. of epinephrine per kgm. (starting at time 345). The injection of an additional dose of 0.1 mgm. veratramine per kgm., at 3, led to signs of awakening and hyperexcitability of the animal and convulsions appeared (at time 380) although lighter in intensity than those previously observed. Intravenous administration of 35 mgm. sodium pentobarbital promptly restored anesthesia.

In the spinal cat (figure 11 A and B) no signs of stimulation of the remaining part of the central nervous system became apparent in the absence of any general anesthetic agent and with a dosage range comparable to that of the experiment (of figure 10) in the anesthetized dog.

IV. *The dissociation of positive inotropic and positive chronotropic actions of epinephrine.* Doses of veratramine which markedly reduce the positive chronotropic effect of epinephrine do not noticeably interfere with the inotropic action on the mammalian heart. This is best demonstrated in the failing heart. Competence in the experiment of figure 12 is increased by the injection of 10 microgm. of epinephrine in spite of the fact that acceleration no longer occurs; increase in output is due entirely to increase in stroke volume. Even 30 microgm. given subsequently did not increase the heart rate except for a period of ten seconds during the first minute after the injection (7). The duration of the positive inotropic action of epinephrine in the failing heart obtained with single doses is of the same order as in the heart not treated with veratramine. After continuous

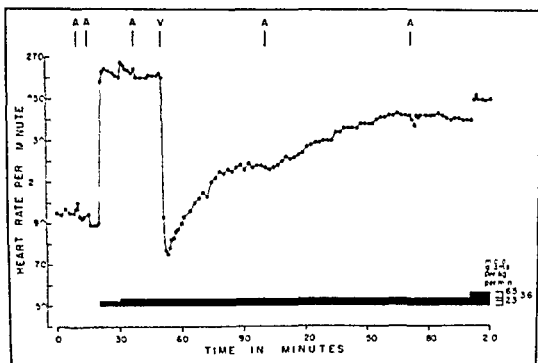


FIGURE 11A THE INHIBITION OF THE POSITIVE CHRONOTROPIC ACTION OF EPINEPHRINE BY VERATRUMINE IN THE PRESENCE OF ATROPINE

Cat, female, 3 kgm. Spinal preparation decerebrated and medulla destroyed under ether anesthesia. At  $V_1$  5 mgm atropine sulfate intravenously. At  $V_2$  0.2 mgm veratramine per kgm intravenously. Black bar continuous infusion of epinephrine (1:5000), calibration on right in microgm per kgm per minute.

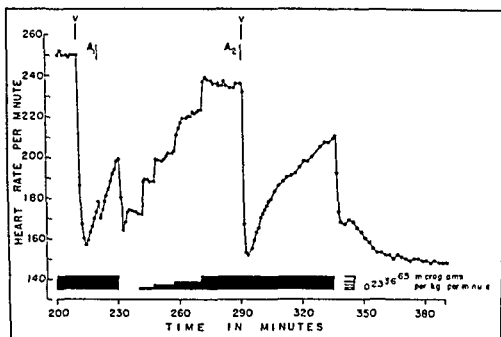


FIGURE 11B THE INHIBITION OF THE POSITIVE CHRONOTROPIC ACTION OF EPINEPHRINE BY VERATRUMINE IN THE PRESENCE OF ATROPINE

Cat female, 3 kgm. Spinal preparation decerebrated and medulla destroyed under ether anesthesia (same experiment as 11A). At  $V_1$  0.2 mgm veratramine per kgm intravenously. At  $A_1$  5 mgm atropine sulfate intravenously. At  $A_2$  10 mgm atropine sulfate intravenously. Black bars continuous infusion of epinephrine (1:5000), calibration on right in microgm per kgm per minute.

infusion of epinephrine the positive motropic effect disappears at approximately the same rate without and with veratramine.

V. *Coronary flow in the HLP.* Accompanying the positive inotropic and positive chronotropic action of epinephrine in the HLP there is an increase in coronary flow. Measurements of coronary sinus outflow by means of a Morawitz cannula

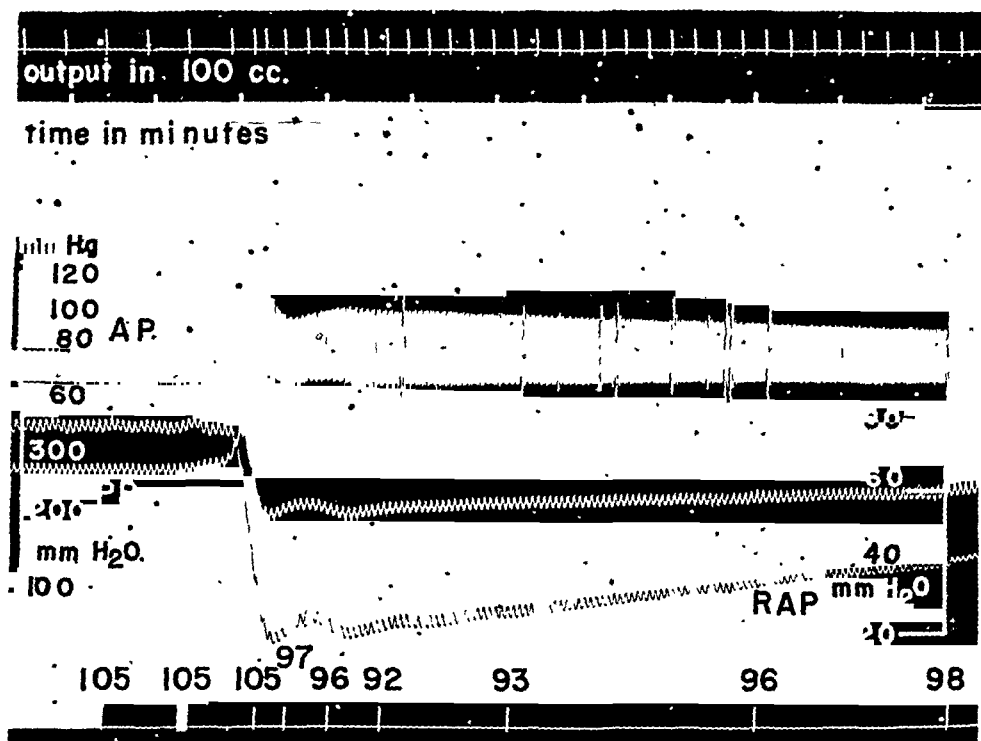


FIGURE 12. THE POSITIVE INOTROPIC ACTION OF EPINEPHRINE IN THE ABSENCE OF POSITIVE CHRONOTROPIC ACTION AFTER VERATRAMINE

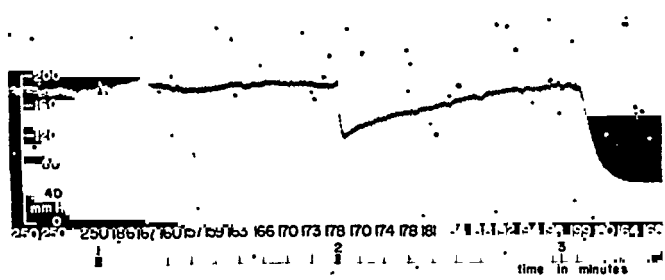
Dog, male, 11.6 kgm. Heart-lung preparation. From top to bottom: Systemic output (total output of left ventricle minus coronary flow) measured by Weese stromuhr in 100 cc/stroke Time in minutes. AP: arterial pressure recorded with Hg manometer, calibration on left in mm. mercury. PP (lower curve on left—middle curve on right): pulmonary pressure recorded with bromoform manometer, calibration on left in mm. water. RAP (middle curve on left, lower on right): right atrial pressure recorded with water manometer, calibration on right in mm. water. Horizontal row of figures: heart rate per minute. 8.5 mgm. veratramine were given in divided doses during 2 hours preceding the epinephrine administration. At the broad signal 10 microgm. of epinephrine were injected close to the heart. Temperature of the blood varied between 38.5 and 38.8° C Total blood volume was approximately 500 cc.

showed that this vascular effect was not abolished by doses of veratramine which largely obviated the cardioaccelerator effect of the administration of single doses or of continuous infusion of epinephrine.

VI. *Vasomotor effect of epinephrine.* In anesthetized dogs and cats and in the spinal and completely pithed cat the blood pressure increase produced by continu-

ous infusion of epinephrine is not consistently and regularly modified to any great extent by doses of veratramine which greatly change the heart rate. Figure 13 shows no change in blood pressure in a spinal cat receiving 0.2 mgm. veratramine per kgm. which decreased the heart rate by 31 per cent, and completely abolished the positive chronotropic action of epinephrine.

Veratramine alone causes a decrease in blood pressure in doses corresponding to those used in these blood pressure experiments. This effect, however, does not become apparent and is insufficient to counteract the vasoconstrictor influence of the concentrations of epinephrine whose cardioaccelerator action can be largely or entirely overcome.



The inhibitory effect of veratramine upon impulse generation is not such as to render the tissue of the pacemaker entirely incapable of responding to epinephrine. High enough concentrations of epinephrine (or nor-epinephrine) are still capable of increasing heart rate.

The rate of degradation of epinephrine does not appear to be modified by the presence of veratramine, since the duration of action of the inotropic effect in the failing HLP is of the same order of magnitude as in the heart not under the influence of veratramine. Likewise, the blood pressure decrease after discontinuation of continuous infusion of epinephrine in the whole circulatory system proceeds at a rate not noticeably different from that observed when no veratramine is present.

2. The specificity of the veratramine effect is emphasized by a comparison with the action of other substances hitherto known as epinephrine antagonists. Ergot, as Dale reported (8), does not abolish the cardioaccelerator effect of epinephrine in doses which "suffice to reverse the effect on the blood pressure". Under the conditions of the present experiments 2 mgm. of dihydroergotamine did not change the heart rate elevated by the continuous infusion of epinephrine. Yohimbine in a dose of 5 mgm. and Priscoline in a dose of 10 mgm. were ineffective. The benzodioxane, 933 F, appears to have no effect on the positive chronotropic action of epinephrine on the heart of the cat (9). It has repeatedly been stated that dibenamine does not antagonize the cardioaccelerator action of epinephrine (10, 11). In the HLP of the dog, Acheson, Farah and French (12) found that as much as 150 mgm. of dibenamine diminished neither the positive chronotropic nor the positive inotropic effect of epinephrine.

3. With the use of veratramine it has become possible to separate the positive chronotropic from the positive inotropic action of epinephrine. The two fundamental elements of the cardiac action of epinephrine can now be studied independently.

The vasodilator action of epinephrine upon the coronary vessels in the isolated, denervated heart (HLP) is not abolished by veratramine in doses which reduce the heart rate to normal when epinephrine is given by continuous infusion, or which prevent an increase in heart rate when epinephrine is given in single injections. It will now be possible to investigate the extent to which the positive inotropic action and the positive chronotropic action participate in the vasodilator response of the coronary vessels.

4. Of the known veratrum alkaloids, jervine has been found to inhibit the positive chronotropic action of epinephrine in a manner similar to veratramine. Its potency is considerably less. Veratrosine, the glycoside of veratramine, and pseudojervine, the glycoside of jervine, also were found to possess the characteristic property. The introduction of glucose, however, modifies the effect. As will be shown in subsequent communications, there are qualitative as well as quantitative differences between the effect of the glycosides and that of their respective aglycones. The ester alkaloid veratridine in a dose of 0.3 mgm. was ineffective. It is impossible to administer larger single doses of this compound on account of its tendency to cause disturbances of rate and rhythm of the heart

beat (13). The alkamine cevine was ineffective in a dose of 40 mgm., while 0.1 mgm. of veratramine under the same conditions caused a definite effect.

5. Chemically veratramine (like jervine) belongs to the group of secondary veratrum bases in which nitrogen is contained in the steroid side chain. Up to the present the published data do not explain all the structural details of this part of the molecule. Veratramine is a benzenoid compound. Possibly ring B is the unsaturated benzenoid ring (2).

#### SUMMARY

Veratramine, a secondary base of the veratrum alkaloid series, antagonizes the cardioaccelerator (or positive chronotropic) action of epinephrine in the isolated, denervated heart (HLP) of the dog, as well as in the whole circulatory system of dogs and cats under anesthesia, or of spinal or completely pithed cats.

The site of action is in the pacemaker tissue of the heart. Atropine in large doses does not modify this veratramine effect.

The action of epinephrine upon contractility (positive inotropic action) is not abolished by doses of veratramine which annul the cardioaccelerator action. It is thus possible to separate the two fundamental elements of the cardiac action of epinephrine.

The vasodilator action of epinephrine upon the coronary vessels of the denervated isolated heart (HLP) of the dog, and the vasopressor action of epinephrine in the whole circulation are not abolished by doses of veratramine which have a very marked effect upon heart rate.

The hitherto known antagonists of epinephrine, which abolish or prevent its vasopressor action, are devoid of the property of veratramine, when they are subjected to study under identical conditions.

Of several other veratrum alkaloids studied, jervine, another secondary base, and the glycosides, veratrosine and pseudojervine, were also found capable of antagonizing the cardioaccelerator effect of epinephrine.

It is a pleasure to acknowledge the help given in these experiments by Dr. Felix Bergmann, Mr. Henry George, and Mr. Arthur Campbell.

#### REFERENCES

1. SAITO, K.: Bull. Chem. Soc. Japan., 15: 22, 1940.
2. JACOBS, W. A., AND CRAIG, L. C.: J. Biol. Chem., 160: 555, 1945.
3. KRAYER, O.: Arch. f. exper. Path. u. Pharmacol., 162: 1, 1931.
4. KRAYER, O., AND MENDEZ, R.: THIS JOURNAL, 74: 350, 1942.
5. STRAUD, W.: Arch. f. exper. Path. u. Pharmacol., 185: 456, 1937.
6. HUNT, R.: J. Exper. Med., 2: 151, 1897.
7. KRAYER, O.: Proc. Soc. Exper. Biol. and Med., 70: 631, 1949.
8. DALE, H. H.: J. Physiol., 34: 163, 1906.
9. HILL, V. T., AND MYERS, G. S.: Am. J. Physiol., 126: 305, 1939.
10. NICKERSON, M., AND GOODMAN, L. S.: THIS JOURNAL, 89: 167, 1947.
11. NICKERSON, M., AND NOMAGUCHI, G. M.: THIS JOURNAL, 95: 1, 1949.
12. ACHESON, G. H., FARAH, A., AND FRENCH, G. N.: Fed. Proc., 6: 305, 1947.
13. MOE, G. K., AND KRAYER, O.: THIS JOURNAL, 77: 220, 1943.

# EFFECT OF DETERGENTS ON VARIOUS STRUCTURES, WITH SPECIAL REFERENCE TO MUSCLE AND GANGLION

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The addition of "Zephiran" to a solution of Carbachol instilled in the eye increases the amount of the choline derivative found in the anterior chamber (1). The same result is obtained when a solution of physostigmine in "Zephiran" is used (2). Von Sallman and Meyer (3) found that several detergents favor the penetration of penicillin into the aqueous humor. With these facts in mind, we thought it of interest to find out if certain detergents can increase the response of skeletal muscle, smooth muscle, and autonomic ganglion to injections of acetylcholine and potassium chloride. Since the available literature contained no data showing whether or not the detergents studied lowered surface tension and facilitated the penetration of certain substances into cells, we decided to make such a study.

**METHODS.** The chronically denervated quadriceps muscle, the superior cervical ganglion, and the nictitating membrane of cats, anesthetized with pentobarbital sodium according to our present technique (4), were used for this study. The doses of acetylcholine and potassium chloride used were 5 to 60 mgm. and 15 to 20 mgm., respectively. Both drugs were dissolved in 0.2 cc. of distilled water and injected into the caudal end of the abdominal aorta.

The detergents used were "Santomerse" (Monsanto)—an alkylarylsulfonate—in doses of 0.25 to 1 cc. of a 1:100 solution; "Merpel" (Dupont)— $R-CH_2-O-SO_3Na$ , where R is a long alcoholic chain—in doses of 0.5 to 1 cc. of a 1:100 solution; "Invadine N" (Ciba)—an alkyl-naphthalylsulfonate—in doses of 0.35 to 1 cc. of a 1:500 solution; "Duponal 189" (Dupont)— $CH_3(CH_2)_{10}CH_2-O-SO_3Na$ —in doses of 0.40 to 0.70 cc. of a 1:1000 solution; and "Zephiran" ("Zobenol" Winthrop)—an alkyl-dimethylbenzylammonium chloride of high molecular weight—in doses of 0.2 to 1 cc. of a 1:1000 solution. All were administered by intra-aortic injection.

**I. ACTION OF DETERGENTS ON SURFACE TENSION AND CELLULAR PERMEABILITY.** *A. Action of detergents on surface tensions.* The changes produced by detergents on the surface tension of distilled water and of cats' serum were studied by the method of Dognon (5) (table 1). All the detergents decreased surface tension in both cases. The fall in surface tension of water was generally parallel to that of serum, except for the much greater effect of "Santomerse" on water than on serum.

*B. Action on cellular permeability.* Two types of experiments were carried out. In one potassium iodide was chosen, although it is slightly less diffusible than acetylcholine or potassium chloride. Three-tenths cc. of a 5 per cent solution of potassium iodide was placed on the conjunctiva of a cat, while the other eye was similarly treated except that the potassium iodide was dissolved in a detergent solution. After fifteen minutes (when "Merpel" was used) and twenty-five

minutes (in the case of the other detergents) 0.5 cc. of aqueous humor was withdrawn and analyzed for iodide. The detergents favored penetration of iodide into the aqueous humor (table 2).

In the other type of experiment, we studied the mortality curve of the frog *Cystignalus bibronii*, when placed in potassium chloride solutions of different concentrations. For each concentration, ten frogs were used. With the same

TABLE 1

*Changes of surface tension of distilled water and blood serum of cats produced by detergents*

	DISTILLED WATER DYNES/CM <sup>1</sup>	BLOOD SERUM DYNES/CM <sup>1</sup>
Distilled water.....	75.*	—
Blood serum of cat.....	—	59.60
Merpel 1:100.....	28.07	31.75
Santomerse 1:100.....	32.45	56.61
Zephiran 1:1,000.....	37.13	—†
Duponol 1:1,000.....	37.25	33.30
Invadine 1:500.....	40.88	42.32

\* According to Dognon.

† Having only a 1:1,000 solution available, we could not determine this.

TABLE 2

*Influence of detergents on the penetration of potassium iodide through the cornea of cats*

DETERGENT	TIME  min.	IODINE IN MG. PER 0.5 CC. AQUEOUS HUMOR*	
		Control	Experiment
Merpel.....	15	0.00	2.6
Santomerse.....	25	0.00	1.9
Zephiran.....	25	0.00	0.2†
Duponol.....	25	0.00	2.1
Invadine.....	25	0.18	1.8

\* Each figure represents the mean of two results.

† Potassium iodide added to a solution of Zephiran produces turbidity.

method, the mortality curve for "Merpel" was determined. The mortality curve for potassium chloride was then determined in the presence of the maximum concentrations of "Merpel" that showed 0 per cent mortality (fig. 1).

The displacement of the mortality curve produced by potassium chloride when used together with "Merpel" is greater than would be expected from a simple summation of effects and suggests that the detergent has increased the penetration of potassium iodide through the skin.

II. ACTION OF DETERGENTS ON CHOLINESTERASE ACTIVITY. Human blood serum was used. The method of Pighini (6) as modified in our department (7), was used for titrations of cholinesterase activity. The error of the method was  $\pm 0.107$



for 2.38 cc. N/200 sodium hydroxide in six titrations of the same serum. Of these detergents, only "Zephiran" inhibited cholinesterase (table 3).

III. EFFECT OF DETERGENTS ON THE RESPONSE OF MUSCLE TO ACETYLCHOLINE AND POTASSIUM CHLORIDE. Repeated intra-aortic injections of acetylcholine were given until uniform muscular contractions were obtained; then, fifteen seconds

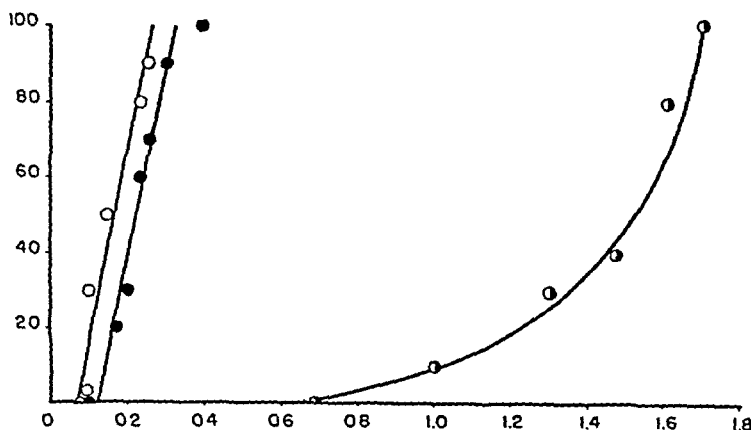


FIG. 1. Action of Mepol on the mortality curve of *Cystignatus bibronii* produced by potassium chloride. Ordinates, percentage mortality; abscissae, logarithm of dose.

● Mortality curve of *Cystignatus* produced by potassium chloride.

○ Mortality curve of *Cystignatus* produced by Mepol.

○ Mortality curve of *Cystignatus* produced by potassium chloride plus Mepol.

TABLE 3  
Anticholinesterase activity of detergents

	EXP. NO.						AVERAGE
	1	2	3	4	5	6	
Normal serum .....	1.64	2.03	1.71	2.13	1.83	1.79	$1.88 \pm 0.242$
Normal serum + Zephiran....	0.62	0.83	0.75	1.25	1.10	1.15	$0.95 \pm 0.1$
Normal serum + Santomerse....	1.95	2.67	1.78	2.10	1.71	2.01	$2.02 \pm 0.137$
Normal serum + Mepol.....	1.99	2.04	1.94	2.07	1.86	2.01	$1.98 \pm 0.025$
Normal serum + Invadine....	1.67	2.10	1.86	2.18	2.05	2.14	$2.00 \pm 0.066$
Normal serum + Duponlo....	1.52	2.46	1.47	1.96	1.76	1.93	$1.84 \pm 0.141$

before the next injection of acetylcholine, 0.5 to 0.6 cc. of a detergent solution was injected.

Under these conditions, all the detergents used increased the response to acetylcholine (fig. 2). Intra-aortic injection of 10 to 15 mgm. of potassium chloride produced a muscular contraction, the response waning with successive injections. When, after repeated injections, the response to potassium chloride had become very slight, a dose of detergent was given approximately 15 seconds before the next dose of potassium chloride. All the detergents increased muscular contractions considerably in such an experiment (fig. 3).

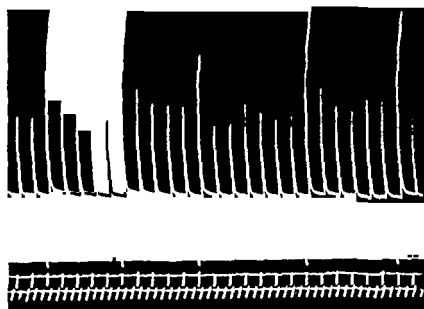


FIG 2 Action of Invadine, Duponol, Zephiran, Santomerse and Merpel on muscular contraction produced by acetylcholine. Muscle with a previous (10 days) aseptic denervation. Signals on upper line (from left to right) 0.6 cc Invadine, 0.6 cc Duponol, 0.6 cc Zephiran, 0.6 cc Santomerse, and 0.6 cc Merpel. Signals on middle line 5 micrograms of acetylcholine. Signals on lower line Time in minutes.

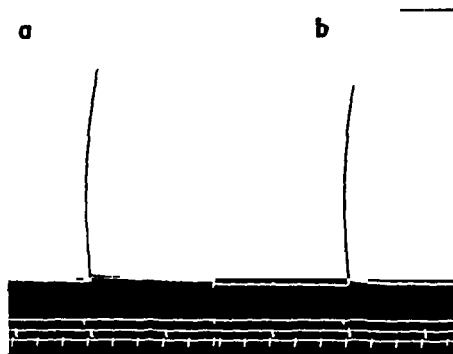


FIG 3 Action of Santomerse and Merpel on muscular contraction produced by potassium chloride.

a. Signals on upper line 0.5 cc Santomerse. Signals on middle line 15 mgm potassium chloride (same in b).

b. Signal on upper line 0.75 cc Merpel.

IV EFFECT OF DETERGENTS ON THE RESPONSE OF THE SUPERIOR CERVICAL GANGLION. This problem was approached in two ways: A. In animals with or

without acute extirpation of the superior cervical ganglia and with or without clamping of the external carotid artery, 20 to 60 mgm. of acetylcholine were injected into the common carotid artery. When a uniform response of the nictitating membrane was obtained, a detergent was injected fifteen seconds before the next dose of acetylcholine. With the exception of "Merpol", which depresses the ganglia and increases the smooth muscle response, the drugs either have no effect or depress the ganglia as well as the nictitating membrane (fig. 4). B. In other animals, pre- and postganglionic cervical sympathetic fibers were stimulated electrically with currents producing tetanic responses. The injection of "Merpol" depressed the nictitating membrane on the side where preganglionic fibers were stimulated.

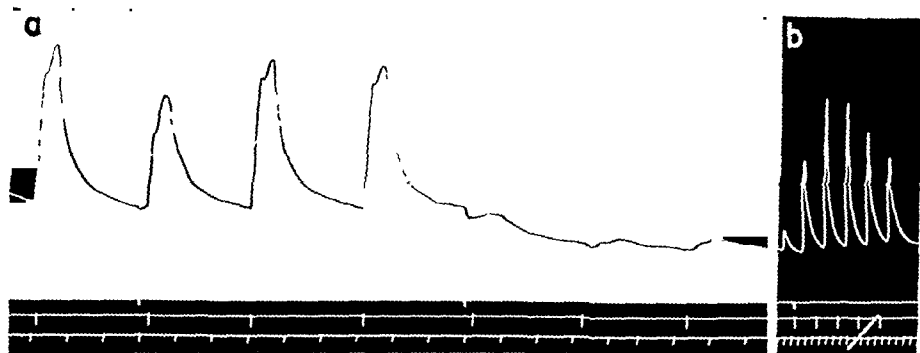


FIG. 4. Action of Merpol on the superior cervical ganglion and nictitating membrane stimulated by acetylcholine.

a. First signal on upper line: 0.5 cc. 1 per cent Merpol; second signal, 0.5 cc. 2 per cent Merpol. Middle line: 60 micrograms acetylcholine injected in carotid artery (external carotid ligated).

b. Another animal with the superior cervical ganglion extirpated. Upper line: 0.5 cc. 1 per cent Merpol. Middle line: 15 micrograms acetylcholine injected into the carotid artery.

*Discussion.* Our results suggest that the increase of muscular response to acetylcholine and potassium chloride produced by detergents is due to an increased permeability of the muscle cell to the first two substances. Torda and Wolff found that agents lowering surface tension increase sensitivity to acetylcholine.

The difference found between the myoneural junction, on one hand, and ganglion and smooth muscle, on the other, is not a question of dosage, since "Merpol", at least, was studied at other concentrations (2:100 to 1:10,000) with the same effect. At a concentration of 1:10,000 "Merpol" loses virtually all its action (table 4).

If detergents increase the response of striated muscle to acetylcholine and potassium chloride through an increase in permeability, they might be expected to act similarly on autonomic ganglion and smooth muscle. However, with the exception of "Merpol", they depress ganglion and smooth-muscle response. The possibility must therefore be considered that these detergents act through additional pharmacological properties. If this is the case, ganglion and smooth muscle may be more sensitive than striated muscle to such addition.

TABLE 4  
*Effect of detergents on the response of ganglion and nictitating membrane to acetylcholine*

With ganglion Concentration	2:100	1:100	5:1000	1:1000	1:2000	1:4000	1:6000	1:8000	1:10000
Merpol	---++	---++	---0	---	---	---	---	---	---
Invadine				---					
Duponol				---+000					
Zephiran				---					
Santomerse		---							
Without ganglion									
Merpol	++++-	++++-		+++					
Invadine									
Duponol			---	---					
Zephiran				---					
Santomerse		---							

- Depressor action.

+ Increase in response.

0 No effect.

Every sign represents one experiment.

Anticholinesterase activity is not responsible for these effects, since "Zephiran" is the only cholinesterase inhibitor used here.

Our results agree with the findings of Reiner (8) for other detergents. The ones we studied, especially a 2 per cent solution of "Merpel"; rapidly produce thrombosis of the common carotid artery.

#### SUMMARY AND CONCLUSIONS

The detergents investigated were found to lower the surface tension of distilled water and of cats' serum and to favor the penetration of substances through the cornea of cats and frogs' skin.

All the detergents used increased appreciably the response of chronically denervated striated muscle to acetylcholine and potassium chloride, but acted as depressors on the superior cervical ganglion and the smooth muscle of the nictitating membrane (except "Merpel", which increased nictitating membrane response).

From the foregoing facts, we conclude that the detergents studied have other pharmacological properties besides that of increasing cellular permeability.

#### REFERENCES

1. O'BRIEN, C. S., AND SWAN, K. C.: Arch. Ophth., 27: 253, 1942.
2. BOYD, J. L.: Arch. Ophth., 30: 512, 1943.
3. VON SALLMANN, AND MEYER, K.: Arch. Ophth., 31: 1, 1944.
4. HUIDOBRO, F., AND AMENABAR, E.: THIS JOURNAL, 84: 82, 1945.
5. DOGNON, A.: *Precis de Physico-Chimie Biologique et Medicale*. Masson et Cie. Paris, 1929.
6. PIGHINI, G.: *Biochim. e Terap. Sper.*, 26: 157, 1939.
7. VALENZUELA, F.: Tesis para optar al titulo de Medico, Santiago, Chile, 1945.
8. REINER, L.: Proc. Soc. Exper. Biol., and Med., 62: 49, 1946.

# BIOCHEMICAL STUDIES ON CHLORAMPHENICOL (CHLOROMYCETIN<sup>1</sup>)

## II TISSUE DISTRIBUTION AND EXCRETION STUDIES\*

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The structure of the new antibiotic chloramphenicol (Chloromycetin<sup>1</sup>) has been established by the work of Rebstock, Crooks, Controulis and Bartz in these laboratories as D(-)-*threo* 2 dichloroacetamido 1 p nitrophenyl 1,3 propanediol (1) The development of a new colorimetric method for aromatic nitro compounds in biological materials (2) has opened the way for studies on the metabolic fate of chloramphenicol, extending the data obtained by others using microbiological assay procedures (3, 4)

From the results presented here it is evident that chloramphenicol is rapidly absorbed, inactivated and excreted In man, about 90 per cent of the drug administered orally is recovered in the urine in 24 hours, principally in the form of inactive metabolic products which retain the aryl nitro group intact Less than 10 per cent of the dose is excreted as unchanged chloramphenicol, confirming earlier observations (4) Evidence is presented for the formation of inactive nitro compounds and aryl amines from chloramphenicol by enzymatic processes The drug is partly excreted in the bile of lower animals, and a large proportion of the total dose is recovered in the intestinal tract The principal metabolic products of chloramphenicol have been isolated and identified and will be described elsewhere (5)

**PROCEDURE** Crystalline chloramphenicol isolated from fermentation sources (6) and synthetic chloramphenicol (7, 8) were used interchangeably in these studies, being identical in chemical structure and properties (1) Dosage figures are given in actual weight of crystal line chloramphenicol administered, rather than in arbitrary units

Microbiological and colorimetric assay procedures were used for the determination of chloramphenicol The microbiological assay method is based on the inhibition of the growth of *Shigella sonnei* as measured by a turbidimetric method (9) The chemical method (2) is based on the reduction of aromatic nitro compounds by titanous chloride, followed by diazotization and coupling of the resulting aryl amine with the Bratton Marshall reagent This determination includes inactive metabolic products of chloramphenicol which contain the nitro group, as well as unchanged chloramphenicol Simultaneous determinations are also made for aryl amines which may be present before reduction, using methods already described (2) In the absence of separate analytical methods for each metabolic product, all results are expressed in terms of "chloramphenicol equivalents"

<sup>1</sup> Parke, Davis and Company trademark for chloramphenicol

<sup>2</sup> Reported in part at the 33rd annual meeting of the Federated Societies for Experimental Biology and Medicine Detroit, Michigan, April, 1949

**EXPERIMENTAL AND DISCUSSION.** *Distribution in Tissues of the Dog, Rat and Guinea Pig.* A series of white rats weighing 160 to 190 gm. were given 100 mgm. of chloramphenicol per kgm. body weight by subcutaneous injection of a 50 per cent propylene glycol solution containing 10 mgm. of antibiotic per cc. The rats were killed in groups of three at intervals of 1, 2, 4 and 10 hours after administration. Tissue samples were removed and stored in a deep-freeze unit until analyzed. Equal weights of corresponding tissues from the animals in each group were pooled and analyzed for total nitro compounds by the titanium reduction procedure (2). Tissues from normal untreated rats were handled in the same manner, and the "blank" values were subtracted from those obtained for corre-

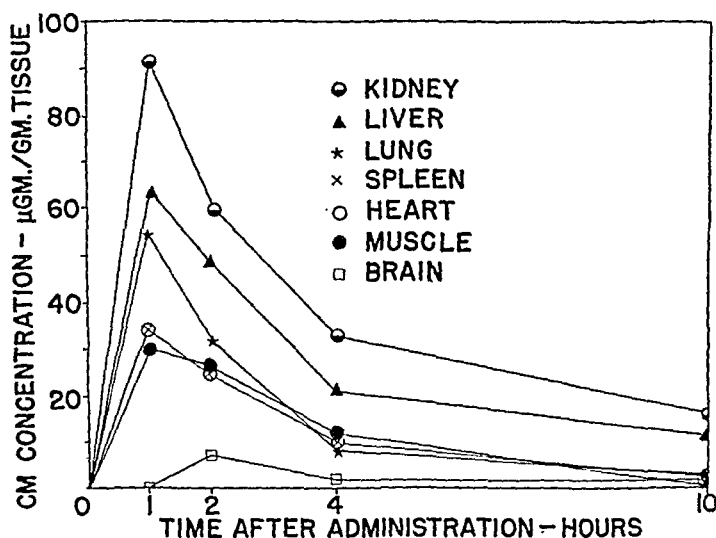


FIG. 1. CONCENTRATION OF NITRO COMPOUNDS IN RAT TISSUES AT DIFFERENT TIME INTERVALS AFTER SUBCUTANEOUS ADMINISTRATION OF CHLORAMPHENICOL

Analytical data were obtained by the titanium reduction procedure on pooled samples of corresponding tissues from three rats killed at each time interval, with corrections being made for normal tissue blanks.

sponding tissues from treated animals. Results are presented in figure 1, expressed as micrograms of chloramphenicol-equivalents per gram of tissue. The greatest concentration of nitro compounds is observed in the kidneys, with progressively lower concentrations in the liver, lung, heart, spleen, muscle and brain.

Similar results were obtained with dogs. Two dogs weighing 5.6 and 5.8 kgm. were each given 35 mgm. of chloramphenicol per kgm. body weight by subcutaneous administration of a 50 per cent propylene glycol solution containing 10 mgm. of the antibiotic per cc. One dog was sacrificed 90 minutes after administration, and the other was sacrificed at three hours. Tissues were collected from each dog immediately after death, and handled in the same manner as described for the rat experiments. The results are presented in table 1. No increase in the concentration of aryl amines was observed over normal levels. The distribution of nitro compounds in the tissues appears to follow the same pattern as in the

rat The ratio of the concentration of nitro compounds in various tissues to that found in plasma is also given in table 1. High ratios for kidney and liver, and

TABLE 1

*Distribution of nitro compounds in the dog*

Dogs given 35 mgm of chloramphenicol per kgm body weight by subcutaneous injection were sacrificed 1.5 and 3.0 hours after administration. Tissues were analyzed for total nitro compounds by the titanium method (2), with results expressed as micrograms of chloramphenicol equivalents per gram of tissue. Corrections were made for normal tissue blanks. The ratio of the nitro concentration in 1 gm of tissue to that found in 1 cc of plasma is also given, with no corrections being made for the apparent drug concentration in plasma water.

TISSUE	1.5 HOURS		3.0 HOURS	
	Concentration (microgm./gm.)	Ratio (tissue/blood)	Concentration (microgm./gm.)	Ratio (tissue/blood)
Kidney	109	5.2	35	2.7
Liver	81	3.9	42	3.2
Lung	43	2.0	13	1.0
Spleen	37	1.8	14	1.1
Heart	32	1.5	11	0.9
Muscle	23	1.1	12	0.9
Brain	15	0.7	8	0.6
Spinal Fluid	7	0.3	9	0.7
Bile	875	41.6	540	41.5
Plasma	21	—	13	—

TABLE 2

*Distribution of nitro compounds and aryl amines in the guinea pig*

Two guinea pigs given 100 mgm chloramphenicol per kgm body weight by the subcutaneous route were sacrificed 90 minutes after administration, and tissues were analyzed for total nitro compounds and aryl amines. Results are expressed as micrograms of chloramphenicol equivalents per gram of tissue, all values being corrected for normal tissue blanks.

TISSUE	GUINEA PIG I		GUINEA PIG II	
	Aryl amines	Nitro compounds	Aryl amines	Nitro compounds
Kidney	110	62	140	100
Liver	52	1	54	4
Lung	1	29	0	26
Spleen	0	10	15	18
Heart	13	9	19	11
Muscle	4	13	4	14
Brain	1	1	2	5
Bile	—	450	—	420
Urine	—	4160	—	3150

low ratios for the brain and spinal fluid indicate a non uniform distribution of the drug throughout the body.



The metabolic fate of chloramphenicol in the guinea pig differs from that observed in the dog in that large amounts of aryl amines are found in the tissues of the former. Data presented in table 2 show that guinea pig liver and kidneys contain relatively low concentrations of nitro derivatives and high concentrations of aryl amines. That this condition is due to enzymatic reduction of the nitro group is supported by *in vitro* experiments described in another section. The rapid formation of aryl amines by the guinea pig may explain the aberrant results observed in this animal with chloramphenicol in toxicity studies and therapeutic tests (10). Although the results presented here show no increase in the urinary excretion of aryl amines by man and dog following administration

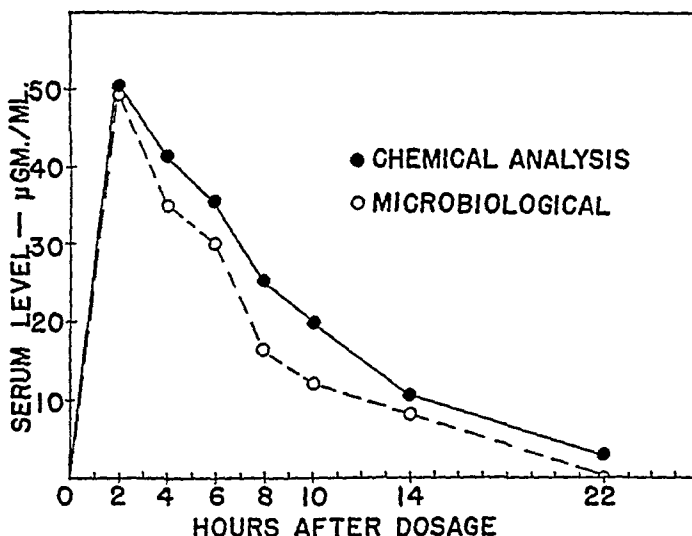


FIG. 2. SERUM LEVELS IN MAN FOLLOWING A SINGLE 3.0 GRAM DOSE OF CHLORAMPHENICOL BY MOUTH

Total nitro compounds were determined by the titanium reduction procedure, and active Chloromycetin was determined by microbiological assay.

of chloramphenicol, an increased excretion of free and conjugated aryl amines has been observed in the urine of the rat, mouse and guinea pig (5).

*Blood Levels of Chloramphenicol in Man.* Following oral administration of chloramphenicol to man, Ley, Smadel and Crocker (4) observed maximum blood levels in two hours by microbiological assay, indicating rapid absorption of the drug from the gastrointestinal tract. These observations are confirmed by the microbiological and chemical data presented here.

A normal adult human subject was given 3.0 gm. of chloramphenicol by mouth in the form of compressed tablets each containing 0.25 gm. of the drug. Blood samples were drawn before administration and at stated intervals thereafter. The serum was separated by centrifugation and a portion was analyzed for active antibiotic by the microbiological method. Trichloroacetic acid filtrates were also prepared for analysis of total nitro compounds by the titanium procedure (2). The results are presented in figure 2, expressed as micrograms of chloramphenicol-equivalents per cc. of serum.

From the data in figure 2, the serum level of chloramphenicol appears to reach a maximum in about two hours, falling off gradually thereafter and returning to normal after 22 hours. The chemical assay values are only slightly higher than the microbiological, indicating that the principal nitro compound in the blood is active chloramphenicol. No significant increase in the aryl amine content of serum was noted in any of the samples. In other studies, a few isolated cases were observed where maximum blood levels were delayed as much as six to eight hours after administration (11); but in the majority of cases human subjects given chloramphenicol by mouth showed maximum blood levels in two to four hours after administration.

Data presented in table 3 summarize a series of observations on human subjects, correlating maximum observed serum levels with the amount of chlor-

TABLE 3

*Relation of maximum serum levels to dose of chloramphenicol in man*

Adult human subjects were given chloramphenicol by mouth, and serum samples were taken at frequent intervals for analysis of total nitro compounds by the titanium reduction procedure. The maximum observed serum levels are related to dosage in the table.

SUBJECT	DOSE	MAXIMUM SERUM LEVEL (MICROGM./CC.)	SERUM LEVEL (MICROGM./CC.)
			RATIO: DOSE (GM.)
A	0.5	9	18
B	0.5	10	20
C	1.5	24	16
D	3.0	43	14
E	3.0	43	14
F	3.0	51	17
Mean serum level per 1 gm. dose.....			17 microgm./cc.

amphenicol (in tablet form) given by mouth. The maximum concentrations appear to be proportional to the size of the oral dose. This can be used as a guide for the prediction of blood levels, but the actual concentrations may deviate considerably from these estimates, being dependent on individual factors of absorption, degradation and excretion.

*Urinary Excretion in Man.* Ley, Smadel and Crocker (4) found that approximately 10 per cent of chloramphenicol given daily by mouth to normal human subjects was recovered in 24-hour urine specimens by microbiological assay. Our results agree with this observation; but *chemical analysis* of the urine for nitro compounds shows that approximately 90 per cent of the drug is excreted in 24 hours, principally in the form of inactive metabolic products which are not detected by microbiological assay.

Two normal adult male subjects were used in these experiments. The bladder was first emptied, and the urine thus voided was used as a control. Subject A received 0.5 gm. of chloramphenicol, and subject B received 1.5 gm. as a single dose in gelatin capsules. Urine

was collected by voluntary emptying of the bladder at stated intervals. Volumes were measured, and aliquots of the urine were stored in a refrigerator until analyzed by the colorimetric and microbiological procedures (2, 9).

The results for subject A, receiving 0.5 gm. of chloramphenicol, are presented in figure 3. The maximum excretion rate as determined by microbiological assay is found 1.5 hours after administration, while the chemical assay for total nitro compounds showed a maximum excretion rate two hours after administration. The quantity of active chloramphenicol recovered in the 24 hour period amounted

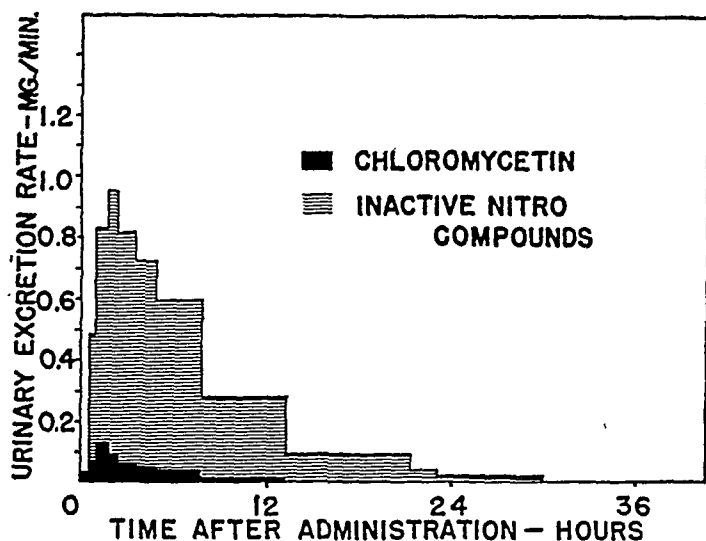


FIG. 3. URINARY EXCRETION OF CHLORAMPHENICOL AND TOTAL NITRO COMPOUNDS IN MAN FOLLOWING ORAL ADMINISTRATION OF 0.5 GRAM CHLORAMPHENICOL

Using the titanium reduction procedure, 87.8 per cent of the administered dose was accounted for in the 24 hour excretion, while 7.5 per cent of the administered dose was recovered by microbiological assay.

to 7.5 per cent of the administered dose, while the total nitro compounds excreted in the same period represented 87.8 per cent of the dose. No increase in the normal excretion of free or conjugated aryl amines was observed in man. The difference between the chemical and microbiological recovery figures is attributed to the presence of inactive metabolic derivatives of chloramphenicol, consisting principally of the glucuronide of chloramphenicol (5). The existence of unchanged chloramphenicol in human urine has also been confirmed by direct isolation and chemical identification of the active antibiotic (5).

The results for subject B, receiving 1.5 gm. of chloramphenicol by mouth, are presented in table 4. The maximum excretion rate for active chloramphenicol again is found to occur slightly ahead of the maximum excretion rate for total nitro compounds. The recovery of active chloramphenicol in the 24 hour urine amounted to 5.6 per cent of the dose, while recovery of total nitro compounds by colorimetric assay accounted for 92.7 per cent of the dose. There was no sig-

nificant increase in excretion of aryl amines over normal levels. Stool specimens collected from this subject for 50 hours after administration showed a slight increase in aryl amines and nitro compounds, accounting for approximately 1 per cent of the administered dose.

A number of observations were made in order to determine whether the urinary excretion rate for nitro compounds is correlated with serum levels. Normal

TABLE 4  
*Urinary excretion of chloramphenicol derivatives in man*

A normal human subject (wt. 73 kgm.) was given 1.5 gm. of chloramphenicol as a single dose by mouth, and urine specimens were collected at stated intervals for analysis by chemical and microbiological assay procedures. Excretion rates are presented in terms of milligrams of chloramphenicol-equivalents per minute, calculated from concentration, urine volume and length of collection period.

TIME OF URINE COLLECTION (HRS. AND MINUTES AFTER DOSAGE)	URINE VOLUME	MICROBIOLOGICAL			TOTAL NITRO COMPOUNDS			ARYL AMINES		
		Concen- tration	Total excreted	Excre- tion rate	Concen- tration	Total excreted	Excre- tion rate	Concen- tration	Total excreted	Excre- tion rate
	cc.	micro- gm./cc.	mgm.	mgm./ min.	micro- gm./cc.	mgm.	mgm./ min.	micro- gm./cc.	mgm.	mgm./ min.
Control--2°30'	195	<1	0	0	0	0	0	20	3.90	0.03
1°00'	105	73	7.7	0.13	600	63	1.05	14	1.47	0.03
1°30'	75	80	6.0	0.20	762	57	1.91	12	0.09	0.03
2°30'	155	94	14.6	0.24	886	137	2.20	18	2.79	0.05
3°35'	84	149	12.5	0.19	2320	195	3.00	22	1.85	0.03
5°30'	90	207	18.6	0.16	3560	347	3.02	70	6.30	0.05
9°05'	141	116	16.4	0.08	2240	316	1.47	42	5.90	0.03
14°00'	180	33	5.9	0.02	1090	196	0.67	44	7.90	0.03
21°30'	180	9.4	1.7	0	350	68	0.15	49	8.50	0.02
24°00'	125	2.6	0.3	0	92	11	0.08	36	4.50	0.03
26°15'	245	<1	0	0	34	8.3	0.06	13	3.18	0.02
45°30'	1140	<1	0	0	11	12.5	0.01	10	11.4	0.01
24 hour excretion (mgm.)			83.7	.....	1390					
Per cent of dose.			5.0	.....	92.7					

human subjects and patients under treatment for urinary tract infections were given chloramphenicol tablets by mouth, and urine samples were collected over fixed time intervals. Blood samples were drawn at the mid-points of the urine collection periods. The results of colorimetric analyses are shown in figure 4, indicating a direct relationship between the urinary excretion rate and serum levels.

**BLOOD LEVELS AND URINARY EXCRETION IN DOGS.** Data on blood levels and excretion rates in dogs gave results similar to those obtained in man except for a smaller recovery in 24 hour urine specimens. A male dog weighing 18.0 kgm. was given 150 mgm. of chloramphenicol per kgm. body weight by mouth in gelatin capsules. Blood samples were taken from the external jugular vein, and urine samples were obtained by catheterizing and completely emptying the bladder at each collection period. The results of chemical and microbiological assay are presented in figure 5.

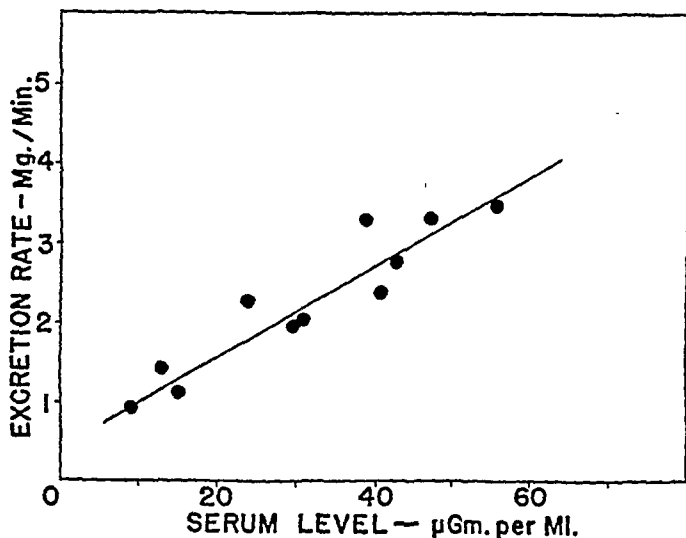


FIG. 4. RELATION OF URINARY EXCRETION RATE FOR TOTAL NITRO COMPOUNDS TO SERUM LEVELS IN MAN

Analytical data were obtained by the titanium reduction procedure and include inactive metabolic products as well as active antibiotic. Serum samples were taken at approximately the midpoints of the urine collection periods.

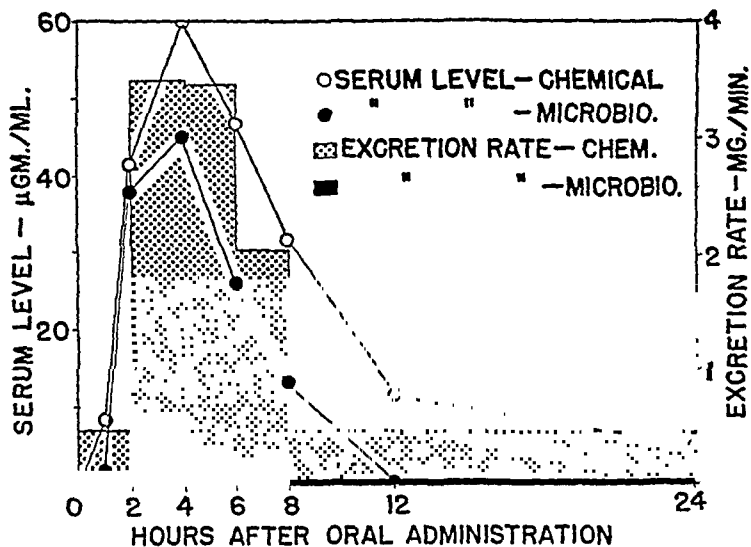


FIG. 5. BLOOD LEVELS AND URINARY EXCRETION RATES FOR CHLORAMPHENICOL AND TOTAL NITRO COMPOUNDS IN A DOG GIVEN 150 MG. OF CHLORAMPHENICOL PER KG. OF BODY WEIGHT BY MOUTH

The amount of chloramphenicol accounted for in 24 hour urinary excretion was 51.7 per cent of the administered dose by the titanium reduction procedure, and 6.3 per cent by microbiological assay.

The excretion rate in dogs appears to be dependent on the blood level, as was also noted in the experiments with man. The colorimetric and microbiological

assay values in serum differ considerably after the first two hours, and inactive nitro compounds are excreted for more than 24 hours although the microbiological assays indicate very low concentrations of active chloramphenicol after twelve hours. However, in contrast with the 90 per cent recovery obtained for 24 hour urinary excretion in man, only 54.7 per cent recovery of total nitro compounds was obtained with this dog. Of the total dose, 6.3 per cent was recovered in the urine as active chloramphenicol. No significant increase in the excretion of aryl amines was noted in the urine; but analysis of feces showed that large amounts of aryl amines were present.

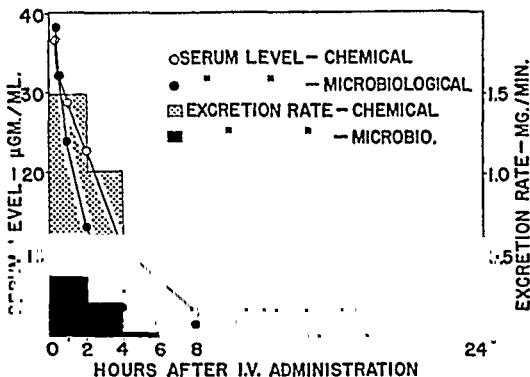


FIG. 6. BLOOD LEVELS AND URINARY EXCRETION RATES FOR CHLORAMPHENICOL AND TOTAL NITRO COMPOUNDS IN A DOG GIVEN 50 MG. OF CHLORAMPHENICOL PER KG. BODY WEIGHT BY INTRAVENOUS INJECTION OF AN 8 PER CENT SOLUTION IN PROPYLENE GLYCOL

The amount of chloramphenicol accounted for in 24 hour urinary excretion was 67.8 per cent of the administered dose by the colorimetric method and 7.6 per cent by microbiological assay.

A similar experiment was conducted with a male dog weighing 16.5 kgm., given a single dose of 50 mgm. chloramphenicol per kgm. *intravenously* as an 8 per cent solution of the antibiotic in propylene glycol. Blood and urine samples were collected as described previously, and analyzed by the colorimetric and microbiological procedures. The results are presented in figure 6. The serum concentration of nitro compounds decreased rapidly, reaching 50 per cent of the initial (15-minute) level in about two hours. Very little active chloramphenicol was found in the urine after six to eight hours, whereas inactive nitro compounds were excreted in the urine for over 24 hours. The recovery of active chloramphenicol in the urine represented 7.6 per cent of the administered dose, while the total nitro compounds in the urine accounted for 67.8 per cent of the dose. There was no significant increase in the aryl amine excretion in urine.

**Renal Clearance.** The renal clearance of active chloramphenicol from the blood-

stream has been calculated from the microbiological assay data in man and dog. In addition estimates have been made of the clearance of inactive metabolic products of chloramphenicol, as represented by the difference between the color-

TABLE 5

*Renal clearance of chloramphenicol and metabolic products in man*

Adult human subjects were given a single dose of 3.0 gm. chloramphenicol in tablet form by mouth. Blood samples were drawn at the mid point of the urine collection periods for analysis by the colorimetric and microbiological assay procedures. No corrections were introduced for protein binding.

SUBJECT	URINE COLLECTION PERIOD (HOURS AFTER ADMIN- ISTRATION)	RENAL PLASMA CLEARANCE (CC./MIN.)	
		Chloramphenicol	Metabolic Products
A	2.0-4.0	26	450
A	4.0-6.0	26	380
B	2.5-3.5	32	190
C	2.5-3.5	19	290
D	6.0-8.5	16	300
E	1.0-2.0	36	260
E	2.0-4.0	13	530

TABLE 6

*Renal clearance of chloramphenicol and metabolic products in the dog*

Dogs were given chloramphenicol orally in gelatin capsules or intravenously as an 8 per cent solution in propylene glycol. Blood samples were drawn at the mid point of the urine collection periods for analysis by the colorimetric and microbiological assay procedures. No corrections were introduced for protein binding.

DOG NO.	DOSE  mgm./kgm.	ROUTE OF ADMIN- ISTRATION	URINE COLLECTION PERIOD (HOURS AFTER ADMIN- ISTRATION)	RENAL PLASMA CLEARANCE (CC./MIN.)	
				Chloramphenicol	Metabolic products
1	50	I.V.	2-4	8	170
2	50	I.V.	0-2	15	210
2	50	I.V.	2-4	12	99
3	150	Oral	2-4	15	260
3	150	Oral	4-6	9	140
4	150	Oral	2-4	15	260
4	150	Oral	4-6	9	150
4	150	Oral	6-8	10	93
5	150	Oral	0-4	29	165

imetric and microbiological assay figures in blood and urine. A summary of the clearance data so obtained is presented in tables 5 and 6, for man and dog, respectively.

The renal clearance of active chloramphenicol appears to be far below the values reported for glomerular clearance of creatinine and inulin in man and dog (12), when no corrections are made for protein binding. Assuming that glomerular

filtration is the principal mechanism for excretion of active chloramphenicol and that no tubular reabsorption occurs, the clearance data presented here would indicate 60 to 80 per cent binding by plasma proteins, which is in agreement with other estimates (3). Experiments on the distribution of chloramphenicol between plasma and red cells in heparinized human blood also show a higher concentration in the red cells than would be expected on the basis of uniform distribution (5).

In contrast with the results for chloramphenicol, the inactive nitro fraction is cleared from the blood at a much greater rate than can be accounted for by simple glomerular filtration. The clearance figures are uniformly high, being from three to five times as great as for creatinine, which would indicate that active tubular secretion is involved (12). This observation does not necessarily apply to all of the metabolic products of chloramphenicol in urine, but it does demonstrate that the principal inactive nitro compounds are excreted far more rapidly than the active antibiotic.

*Excretion in Bile and Feces.* Although the urinary excretion of chloramphenicol derivatives accounts for approximately 90 per cent of the drug in man (table 4), low recoveries from urine are obtained with the dog and other animals. The possible importance of the bile as a route of excretion is indicated by the high concentration of nitro compounds found in the bile of the dog and guinea pig (tables 1 and 2). Since the bile enters the small intestine, it would be expected that large amounts of nitro compounds would be found in the intestinal tract following parenteral administration of chloramphenicol.

A series of white female rats weighing 160 to 190 gm. were given 100 mgm. chloramphenicol per kgm. body weight by subcutaneous injection, using a 50 per cent propylene glycol solution containing 10 mgm. of chloramphenicol per cc. The rats were placed in a metabolism cage over a funnel, using wire screens to separate the feces from the urine. No food was given during the course of the experiment, and there was practically no contamination of the urine with feces. The rats were sacrificed at 4, 8, 12 and 17 hours after administration. The bladders were washed out with saline to insure complete collection of urine. The intestinal tract was dissected out and the entire contents carefully washed out into a beaker, volumes adjusted to 100 cc., and the mixtures homogenized in a Waring Blender. Twenty-five cc. of a 15 per cent trichloroacetic acid solution were then added and the mixtures were filtered. The urine and intestinal tract washings were analyzed separately for nitro compounds and aryl amines. The results are presented in table 7, corrected for normal urine and intestinal contents blanks obtained with untreated rats.

From the results in table 7 it is evident that large amounts of nitro compounds are excreted into the intestinal tract, accounting for as much as three-quarters of the administered dose in eight to twelve hours. That the bile is the principal route of excretion into the intestinal tract is shown by the data in table 8. The intestinal tract of a rat was tied off at various levels and the animal given chloramphenicol by subcutaneous administration. After four hours, the rat was killed and the contents of each section of the intestinal tract were analyzed for nitro compounds and aryl amines. The two-inch section below the pylorus, containing all of the bile excreted into the gut, was found to contain practically all of the nitro compounds found in the intestinal tract.

Another point to be noted is that nitro compounds predominate in the in-



testinal tract during the first four hours, following which the aryl amines increase in concentration and the nitro compounds decrease until practically none remain after seventeen hours (table 7). The aryl amines were found to be concentrated

TABLE 7

*Excretion of chloramphenicol in the urine and intestinal tract of the rat*

A series of rats were given 100 mgm. chloramphenicol per kgm. body weight by subcutaneous injection, and killed at different time intervals after administration. Analyses were made for total aryl amines (free + conjugated) and for total nitro compounds in the urine and intestinal tract. Results are expressed as milligrams of chloramphenicol-equivalents, corrected for normal blanks.

	COLLECTION PERIOD (HOURS AFTER ADMINISTRATION)			
	4	8	12	17
<i>Urinary Excretion:</i>				
Aryl amines.....	0.10	0.20	0.28	0.21
Nitro compounds.....	2.88	3.72	2.92	4.29
Total.....	2.98	3.92	3.20	4.50
Per cent of dose.....	17.7%	21.5%	19.3%	24.3%
<i>Intestinal Tract:</i>				
Aryl amines.....	0.47	2.58	3.30	7.54
Nitro compounds.....	9.83	11.65	9.43	0.72
Total.....	10.30	14.23	12.73	8.26
Per cent of dose.....	61.4%	75.8%	76.8%	44.6%

TABLE 8

*Excretion of nitro compounds in the intestinal tract of the rat*

A rat weighing 168 gm. was anesthetized with ether and the intestinal tract was ligated at various points. The rat was given 16.8 mgm. of chloramphenicol by subcutaneous administration, and sacrificed four hours later. The contents of each section of the intestinal tract were analyzed for nitro compounds by the titanium reduction procedure.

SECTION ANALYZED	NITRO COMPOUNDS (MGM. CHLORAMPHENICOL-EQUIVALENTS)	PER CENT OF DOSE
Stomach.....	0.06	0.4
First 2" of intestine (including bile ducts).....	5.10	30.3
Upper small intestine.....	0.01	0.1
Lower small intestine.....	0.03	0.2
Caecum.....	0.03	0.2
Large intestine.....	0.03	0.2
Total.....	5.26	31.4

primarily in the caecum. Washings of the intestinal tract incubated at 38° C. overnight showed a two-fold increase in the aryl amine content with a corresponding decrease in nitro content. It is therefore probable that the bacterial flora plays an important role in the degradation of the nitro derivatives of chlor-

amphenicol. This is supported by the observations of other workers in this laboratory on the formation of aryl amines from chloramphenicol by bacterial reduction of the nitro group (13).

In contrast to the high concentrations of aryl amines and nitro compounds in the intestinal tract of the rat, only negligible amounts were found in stool specimens collected from man. To study the excretion of nitro compounds in the bile of man, one patient having an external biliary fistula was given 1 gm. of chloramphenicol by mouth, and complete collections of urine and bile were made there-

TABLE 9

*Excretion of chloramphenicol products in urine and bile of man*

One gram of chloramphenicol was given by mouth to a human subject with an external bile fistula. Analyses of urine and bile are given in terms of chloramphenicol-equivalents. Recovery figures are given as per cent of the administered dose.

	TIME OF COLLECTION FOLLOWING ADMINISTRATION (HOURS AND MINUTES)	VOLUME	COLORIMETRIC		MICROBIOLOGICAL	
			microgm./cc.	mgm.	microgm./cc.	mgm.
Urine:	2°10'	400	0	4.4	3.1	1.24
	3°00'	90	123	11	46	4.14
	4°30'	85	272	23	38	3.23
	6°	185	334	62	27	5.0
	7°30'	285	360	103	27	7.7
	9°	180	450	81	26	11.7
	24°	730	730	533	39	28.5
				817 mgm. =81.7%		61.5 mgm. =6.15%
Bile:	3°	130	8	1.0	1	0
	6°	130	43	5.6	2.8	0.36
	9°	65	38	2.5	1.8	0.12
	21°	535	33	17.7	1.6	0.89
				26.8 mgm. =2.7%		1.37 mgm. =0.14%

after. The results of analysis presented in table 9 show that 81.7 per cent of the total dose was recovered in the urine in 24 hours, while only 2.7 per cent of the dose was accounted for in the bile. The possible reabsorption of nitro compounds from the bile in the gall bladder of man has not been investigated, but is suggested as a possible explanation for the low results obtained in man (14).

**INACTIVATION OF CHLORAMPHENICOL BY TISSUE ENZYMES.** The influence of tissue enzymes on chloramphenicol was studied by incubating the antibiotic with minced rat liver *in vitro*. One-gram portions of liver were weighed out, minced by chopping with a razor blade, and suspended in 5 cc. of various buffer solutions as shown in table 10. One cc. of a solution containing 940 microgm. of chloramphenicol was then pipetted into each buffer, and the

samples were incubated at 38°C. for one hour with continuous shaking. Controls were also set up for each buffer without any liver added to check the stability of chloramphenicol under these conditions. After incubation, the samples were homogenized in a modified Potter-Elvehjem apparatus, volumes were adjusted to 20 cc. by the addition of water, and proteins were precipitated by the addition of 5 cc. of 15 per cent trichloroacetic acid. Filtrates were analyzed for total nitro compounds and aryl amines by the colorimetric method (2), and for active chloramphenicol by microbiological assay (9). The results are presented in table 10.

From these results it is evident that the greatest loss of antibiotic activity occurs at pH 7 to 8, amounting to approximately one-third of the initial concentration of chloramphenicol. The major portion of this is represented by the formation of inactive nitro-containing derivatives of chloramphenicol, as evidenced by the difference between the colorimetric and microbiological assay figures (table 10). In addition there is a significant increase in aryl amines, occurring to the

TABLE 10

*Action of rat liver enzymes on chloramphenicol*

Chloramphenicol was incubated for 1 hr. at 38°C. with minced rat liver in 0.1 M acetate (pH 4-5-6), 0.2 M phosphate (pH 7) and 0.2 M borate buffers (pH 8-9). Analytical results are expressed as microgm. of chloramphenicol-equivalents per cc. of deproteinized filtrate.

INITIAL pH	TOTAL NITRO COMPOUNDS (COLORIMETRIC)	ACTIVE CHLORAM- PHENICOL (MICROBIOLOGICAL)	INACTIVE NITRO COMPOUNDS (BY DIFFERENCE)	ARYL AMINES (COLORIMETRIC)
4.0	38.0	35.4	2.6	0.8
5.0	35.2	30.6	4.6	0.6
6.0	35.9	30.6	5.3	1.5
7.0	33.2	25.2	8.0	4.0
8.0	36.0	25.0	11.0	2.2
9.0	36.3	29.0	7.3	0.7

greatest extent in the pH 7 sample. The inactivation of chloramphenicol may involve hydrolysis at the amide linkage in the side chain, since there is evidence or the presence of a small amount of the hydrolysis product in urine (5).

The formation of inactive nitro compounds and aryl amines from chloramphenicol was also observed with rat kidney and with guinea pig liver and kidney. The reducing action was inhibited by the addition of cyanide and hydrogen sulfide, and completely eliminated by heating the tissue preparations to 60° C. for five minutes. The formation of aryl amines from chloramphenicol is not unexpected on the basis of the observations of Kohl and Flynn with nitrobenzoic acid (15).

We wish to express our thanks to Dr. E. C. Vonder Heide, Dr. O. M. Gruhzt, Dr. T. Reutner and Dr. G. Chittenden for their suggestions and kind assistance at various stages of the experimental work; and to Dr. John Schwab, Mr. Dwight Joslyn and Mrs. Margaret Galbraith for conducting the microbiological assays.

## SUMMARY

Chloramphenicol is rapidly absorbed, metabolized and excreted. In man the major route of excretion is by way of the kidneys, with approximately 90 per cent of the administered dose accounted for in 24 hours. Lesser amounts are excreted in the urine of lower animals. The bulk of the excreted drug is in the form of an inactive nitro compound, and less than 10 per cent of the administered dose is excreted unchanged. Renal plasma clearance figures indicate that chloramphenicol is largely excreted by glomerular filtration, while the inactive metabolic products appear to be excreted mainly by tubular secretion.

In the rat, parenteral administration of chloramphenicol results in excretion into the intestine by way of the bile, principally as inactive nitro compounds. These may be partly reabsorbed, or reduced in the intestine to form aryl amines which are excreted in the feces. Inactivation of chloramphenicol occurs *in vitro* with minced liver preparations, with the production of inactive nitro compounds and small amounts of aryl amines. The distribution of chloramphenicol in the tissues is not uniform, with high concentrations being found in the liver and kidneys and low concentrations in the brain and spinal fluid.

## REFERENCES

1. REBSTOCK, M. C., CROOKS, H. M., CONTROULIS, J., AND BARTZ, Q. R.: J. Am. Chem. Soc., in press, 1949.
2. GLAZKO, A. J., WOLF, L. M., AND DILL, W. A.: Arch. Biochem., in press, 1949.
3. SMITH, R. M., JOSLYN, D. A., GRUHZIT, O. M., McLEAN, I. W., PENNER, M. A., AND EHRLICH, J.: J. Bact., 55: 425, 1948.
4. LEY, H. L., SMADEL, J. E., AND CROCKER, T. T.: Proc. Soc. Exper. Biol. and Med., 68: 9, 1948.
5. GLAZKO, A. J., AND DILL, W. A.: Unpublished Observations.
6. BARTZ, Q.: J. Biol. Chem., 172: 445, 1948.
7. CONTROULIS, J., REBSTOCK, M. C., AND CROOKS, H. M.: J. Am. Chem. Soc., in press, 1949.
8. LONG, L. M., AND TROUTMAN, H. A.: J. Am. Chem. Soc., in press, 1949.
9. JOSLYN, D. A., AND GALBRAITH, M.: J. Bact., 54: 26, 1947.
10. GRUHZIT, O. M.: Personal Communication.
11. CHITTENDEN, G. E. et al.: J. Urology, in press, 1949.
12. SHANNON, J. A.: Ann. N. Y. Acad. Sci., 44: 455, 1943.
13. SCHWAB, J. L., AND SMITH, G. N.: Unpublished Observations.
14. ROUS, P., AND McMASTER, P. D.: J. Exper. Med., 34: 47, 1921.
15. KOHL, M. F., AND FLYNN, L. M.: Proc. Soc. Exper. Biol. and Med., 47: 470, 1941.

## THE ANTIFILARIAL ACTION OF CYANINE DYES

### II. SELECTION OF 1'-ETHYL-3, 6-DIMETHYL-2-PHENYL-4-PYRIMIDO-2'-CYANINE CHLORIDE (#863) FOR FURTHER STUDY AS A POTENTIAL ANTIFILARIAL AGENT<sup>1</sup>

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The relative antifilarial activity of a large number of cyanine dyes against the filarial worm, *Litomosoides carinii*, of the cotton rat has been reported in a previous publication (1). Twelve compounds were selected from this larger group for further study because of their marked antifilarial action, *in vivo* and *in vitro* the ultimate objective being a clinical trial in human filariasis of a compound combining the features of high activity and relatively low toxicity. The twelve compounds are listed by number in table 1, which summarizes the pertinent data relating to their high therapeutic indices when tested in cotton rats infested with *L. carinii*, and to their marked inhibitory effect on the oxidative carbohydrate metabolism of this filariid *in vitro*. Details of the studies on which this summary is based were presented earlier (1, 2) along with the structural formulae of the compounds, and a discussion of the relation of chemical constitution to antifilarial effect.

A. *The chemotherapeutic action of the cyanines following oral administration.* An early step in the continued investigation of these compounds consisted in a study of their chemotherapeutic effect against *L. carinii* in the cotton rat using other than the intraperitoneal route.

In some experiments the drugs were mixed homogeneously with a finely powdered diet<sup>2</sup> at levels ranging from 0.06 to 1.0 per cent, the diet being fed to infested rats *ad libitum* (3). With these cups a minimum of wastage was encountered, making it possible to measure the daily food consumption, and to calculate the daily drug intake of the individually housed rats with a considerable degree of accuracy. In other experiments, the drugs were administered, in the form of aqueous solutions, by stomach tube under light ether anesthesia.

Briefly stated, ingestion of compounds #348, #863 and #965 resulted in only occasional cures, even when the drug content of the diet was very high. Actively motile worms were recovered at autopsy from the pleural cavities of rats whose average daily consumption of #348 or #863 over a period of fourteen days ap-

<sup>1</sup> The work described in this series of papers was done, in part, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Western Reserve University (August 1, 1944 to October 31, 1945); in part, under a contract between the Office of the Surgeon General, U. S. Army (November 1, 1945 to December 31, 1946); and in part, with the aid of a grant from the U. S. Public Health Service (since January 1, 1947).

<sup>2</sup> General Mills' "Checkers", finely powdered.

proximated 100 mgm. per kgm. of body weight. Number 965 cured two rats at this dose level, but failed to cure other animals who had consumed half of this amount over the same period. By stomach tube, five daily doses of 100 mgm. of #965 per kgm. cured all of three rats treated; less intensive treatment with #965, and also with #348 and #964, consisting of three to five daily doses ranging in size from 20 to 80 mgm. per kgm., failed to cure twelve of sixteen animals treated.

These data are too limited to permit any conclusions with regard to the relative efficacy of the various drugs used. However, a marked decrease in the curative effect of all the drugs, as a result of the change from the intraperitoneal to the oral route of administration, is very obvious when the above figures are compared

TABLE 1

*The relative antifilarial activity, against L. carinii of the cotton rat, of the most active members of a large series of cyanine dyes (1)*

CHEMOTHERAPY CENTER #	MOLECULAR WEIGHT	CHEMOTHERAPEUTIC ACTIVITY <i>in vivo</i> ; DRUG ADMINISTERED I.P. ONCE DAILY FOR 5 DAYS			"IN VITRO INDEX" <sup>†</sup>
		Min. cur. dose*	Max. tol. dose.	Therapeutic index	
		mgm./kgm.	mgm./kgm.		
348	382	0.2	2.0	10	1.0
713	374	0.8	8.0	10	0.25
963	395	0.3	4.0	13	1.5
964	410	0.15	2.0	13	0.8
965	436	0.15	2.0	13	0.5
943	368	0.40	4.0	10	1.0
967	398	0.40	6.0	15	1.0
824	409	1.60	24.0	15	0.25
798	363	0.80	8.0	10	0.50
871	345	0.15	2.0	13	0.80
835	410	0.40	4.0	10	1.0
863	390	0.40	4.0	10	2.0

\* Autopsy was performed 48 hours after the last dose; worms removed at this time were found to be permanently immotile at these dosage levels.

† Relative inhibitory effect on oxygen uptake. The inhibitory effect of the reference standard (# 348) was demonstrable with concentrations of 1:25,000,000 (2).

with those of table 1. In the few cases where cures followed oral medication, the dose used was at least several hundred times as great as that which produced consistent cures following intraperitoneal injection. This suggested that the clinical therapy of filariasis by oral medication with the cyanines would not be practicable. The additional observation that these compounds caused marked gastric irritation in dogs, as evidenced by severe nausea and vomiting following oral administration in solution or in gelatin capsules, led to the rejection of this route of administration in subsequent studies.

*B. The chemotherapeutic and local irritant action of the cyanines following subcutaneous administration.* Treatment of infested cotton rats by the subcutaneous route revealed a pronounced tendency of the cyanines to cause severe local injury; only #964, #965 and #863 were relatively free of this proclivity.

For administration by the subcutaneous route the drugs were dissolved in 5 per cent glucose solution. Although solution in physiological saline could be attained readily if the samples were dissolved in an amount of alcohol equal to 1.0 per cent of the final volume, with many compounds precipitation occurred gradually over a period of five to ten minutes following dilution to final volume with saline. A few compounds (e.g., #863) did not show this effect, and none of them was precipitated by glucose.

In testing these compounds for local irritant effect, 0.5 cc. of an approximately neutral solution of the drug was injected into cotton rats, under the skin of the back; after 24 hours the skin over the injection sites was incised for gross examination of the area. Except in the cases of the four compounds mentioned above, sites exposed to concentrations as low as 0.2 mgm. per cc. showed marked hyperemia and edema, and in many cases softening and liquefaction of tissue suggestive of necrotic change. No local reactions were observed in control rats injected with glucose solution. Compounds #963, #964, #965 and #863 produced no such changes at low concentrations, although the first three compounds named produced slight but definite tissue change at a level of 1.0 mgm. per cc. Even at 2.0 mgm. per cc., compound #863 produced only mild hyperemia and edema. The subcutaneous tissues of dogs were more sensitive than those of the cotton rats. Concentrations of 0.5 mgm. per cc. of #963, #964, and #965 produced severe hyperemia, edema, softening and liquefaction of tissue. The irritant effect of #863 was definitely less marked, but similar changes could be produced when the concentration was raised to 1.0 mgm. per cc.

The results of chemotherapeutic studies with #863 and #965, using the subcutaneous route of administration, also were not encouraging. The minimum curative doses were five to ten times as great as when the intraperitoneal route was used, and the margin of safety was less than half as great. The increase in the amount of drug required for cure, when given subcutaneously, may be accounted for in part by the retention of the drug, as evidenced by the marked staining of the tissues, at the sites of injection. Furthermore, the severity of damage to local tissues was sufficient to suggest its contribution to the apparent systemic toxicity of the drug. The intramuscular route offered no advantage over the subcutaneous, with respect to either chemotherapeutic activity or local and systemic toxicity.

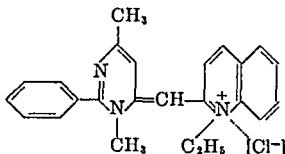
Finally, on the basis of the above findings, it would seem likely that very dilute solutions of these drugs—e.g., less than 0.5 mgm. per cc.—would have to be used to avoid severe local reactions following subcutaneous or intramuscular injection into human beings. The volumes involved in doses as low as 0.5 mgm. per kgm. of body weight therefore would be so large as to be impracticable. The intravenous route of administration thus remained as the only alternative for further study.

*C. The chemotherapeutic action of the cyanines following intravenous administration.* In selecting compounds of proved activity for these experiments the field of choice was limited purposely to those which were least capable of producing local tissue injury. The possibility of producing injury, by intravenous administration,

to the intimal surfaces of veins, with resultant inflammation, sclerosis and thrombosis, was considered, as was the hazard of perivascular injury following improper placement of the drug. Since #965 and especially #863 produced the least evidence of local tissue injury following subcutaneous injection, they were selected for preliminary experiments using the intravenous route. Only #863 was retained for more exhaustive study for a number of reasons.

Earlier in these studies it was observed that #348, the original compound studied, produced a marked drop in blood pressure when administered intravenously to anesthetized dogs and rabbits. Studies with #965 and #863 revealed a similar effect, qualitatively, but also showed that the hypotensive action of the latter compound was much less marked than that of either #348 or #965. Collapse and death due to circulatory depression occurred in unanesthetized dogs within a few hours after the intravenous injection or slow infusion of 5.0 mgm. of #965 per kgm. of body weight. Intravenous injection or infusion of #863 produced no untoward signs, even at 10.0 mgm. per kgm., with the exception of nausea and vomiting. Only when the dose was increased to 15.0 mgm. per kgm. did some animals become progressively weaker after the first 24 hours, a condition followed, during the ensuing 48 hours, by muscular twitchings, rigidity, convulsions, hyperpnea and, eventually death.<sup>3</sup> Therefore, on the basis of both local and systemic toxicity, #863 appeared to offer definite advantages over #965.

Before the latter compound was eliminated finally, a limited comparison was made of the relative chemotherapeutic activity and toxicity of the two compounds in infested cotton rats using the intravenous route of administration. The curative activity and toxicity of #863 was studied eventually in greater detail, as illustrated by table 3 and a subsequent section of this report. Suffice it to say at this time that three to four times as much of #863 was required to produce death, following both single and repeated injections, as was the case with #965. The minimal dose required to produce cure, on repeated injection, was also greater with #863, but its margin of safety was at least as great as that of the other compound. Although the results actually suggested #863 to be slightly superior to #965 in regard to margin of safety, an insufficient number of these expensive animals were treated with the latter compound to establish definitely this small point of difference. Since #863 already had been found to possess advantages in other respects, it was decided not to belabor this point and to devote further work to a more extensive investigation of this one compound.



1'-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2'-cyanine chloride (#863)

<sup>3</sup> The basis of these signs of toxicity was later found to be a depression of kidney function and uremia. A more detailed investigation of this nephrotoxic effect of high doses of #863 is in progress (11).



In earlier studies, when it was established that #863 was capable of producing cures following its intravenous administration to cotton rats harboring *L. carinii*, the question arose as to what general type of therapy schedule would allow this compound to exert its antifilarial effect with the greatest efficiency and safety. In re-examining the data obtained from curative assays using the intraperitoneal route (table 2), it appeared that the therapeutic efficiency of these dyes did not depend on frequent dosage designed to maintain a high level of drug in the blood and tissue fluids, and, in this case, particularly in the pleural fluid surrounding the parasites. The therapeutic indices of #965 and #863, given intraperitoneally, were actually lower when the drugs were given at 8-hour intervals for 18 doses than when given at 24-hour intervals for 5 doses. As is shown in table 2, #348 appears to have the same margin of safety and therapeutic index by both sched-

TABLE 2

*Chemotherapeutic activity of several cyanine dyes against the filarial parasite, L. carinii, of the cotton rat following intraperitoneal administration*

COMPOUND NO.	NO. OF DOSES	NO. OF RATS PER DOSE	INTERVAL LAST DOSE TO AUTOPSY	MIN. CURATIVE DOSE		MAX. TOLERATED DOSE		THERAPEUTIC INDEX	"IN VITRO INDEX"
				Single	Total	Single	Total		
			hours	mgm./kgm.	mgm./kgm.	mgm./kgm.	mgm./kgm.		
348	18	3	48	0.10	1.8	1.0	18.0	10.0	1.0
	5	3	48	0.20	1.0	2.0	10.0	10.0	
	3	3	96	0.30	0.9	2.0	6.0	6.7	
	1	3	168	1.35	1.35	4.0	4.0	3.0	
965	18	5	48	0.10	1.8	0.5	9.0	5.0	0.5
	5	10	48	0.15	1.0	2.0	10.0	13.3	
863	18	5	48	0.10	1.8	0.5	9.0	5.0	2.0
	5	10	48	0.40	2.0	4.0	20.0	10.0	

ules. However, as mentioned in the previous paper (1), the toxicity tests with #348 for the 8-hour schedule were performed with uninfested trapped Florida cotton rats, while those for the 24-hour schedule, as well as for all subsequent toxicity tests reported, were done with cotton rats of a single strain obtained from a breeder<sup>4</sup>. Because the wild variety tolerated the drug somewhat better than the domesticated, it is very probable that, in the case of #348 also, the therapeutic efficiency was greater when the daily injection schedule was used.

Furthermore, examination of the data of table 2 shows that the total dose required to produce cures did not differ as greatly, from one schedule to another, as might have been expected if the maintenance of a constant concentration of drug in the pleural fluid were a critical factor in the chemotherapeutic action of the drug. In a subsequent paper (4), data will be presented to show that in dogs, at least, only a very small proportion of an injected dose of #863 remains in the tissue 24 hours after administration; yet in cotton rats the extension of the

<sup>4</sup> Tumble brook Farms, Brant Lake, New York.

dosage interval from 8 to 24 hours did not impair the efficiency of the drug and, in fact, appeared to result in a wider margin of safety. In the case of #348, cures were obtained with three doses of 0.3 mgm. per kgm. administered intraperitoneally at intervals of 24 hours or even with a single dose of 1.35 mgm. per kgm., if the interval between the last dose and the autopsy were prolonged beyond the usual 48-hour period. The low therapeutic index with the single dose schedule was due, of course, to the approximation of the dose to the single or acute minimal lethal dose of the drug.

It became apparent that the total dose administered, rather than the magnitude of the individual doses and the duration of the intervals between them, merited consideration as the critical factor in the chemotherapeutic action of the cyanines. Such an hypothesis is reminiscent of the observations of Eagle *et al.* (5), who demonstrated the existence of such a situation with respect to the chemotherapeutic action of oxophenarsine in the syphilis of rabbits. Swinyard, Hirschfelder and Wright (6) observed a similar phenomenon with oxophenarsine in their studies on rats infected with *Trypanosoma equiperdum*.

The cyanine dyes are taken up in high concentration by the filarial worm, *L. carinii*. When placed in very dilute solutions of #863 or one of the other active cyanines, the worms rapidly and progressively are stained, the intensity of the color being much greater than that of the surrounding medium. This probably accounts for the fact that for efficient chemotherapeutic results frequent injections of the cyanines are unnecessary. It may also explain the increase seen in the chemotherapeutic efficiency of the drug when the interval between the last dose and the removal of the worms from the host was prolonged. This phenomenon, observed particularly in experiments where the number of doses was small and the interval between them short, is attributed to the fact that more time was allowed for the drug, present in or on the parasite, to exert its lethal effect. In view of these findings, and by analogy with the reports of Eagle *et al.* (5) and Swinyard *et al.* (6), it seemed likely that the cyanines should be capable of exerting a filaricidal effect with a variety of dosage schedules in which the number and magnitude of the individual doses, and the frequency of administration, could be varied over a rather wide range, provided that the total dose did not fall below some critical level, or did not produce a lethal effect on the host. It was on this basis that various dosage regimes were devised for the treatment, by the intravenous route, of cotton rats infested with *L. carinii*.

For each injection the animals were lightly anesthetized and restrained on their backs by means of string-ties extending from the extremities. The head was immobilized in extension by means of a loop of string placed over the upper incisor teeth. Injections were made into the jugular vein at a rate of approximately 0.2 cc. per minute, the maximum volume injected being 0.5 cc. The 0.5 cm. incision made over the external jugular vein to facilitate injection, was closed after each dose by a single silk suture; signs of infection were not encountered.

The various dosage regimes used, and the results obtained, are recorded in table 3. When single doses of 1.0 mgm. of #863 per kgm. were used, many cures were obtained with three doses administered at intervals of either 24 hours, 72

hours, or seven days. When the individual dose was less than 1.0 mgm. per kgm., using the daily injection schedule, more failures occurred. When treatment failures were encountered on the 72-hour schedule, using four doses each of 1.0 mgm. per kgm., the individual dose was raised to 1.25 mgm. per kgm. in another group of cotton rats, all of which yielded only dead worms at autopsy. If it is postulated

TABLE 3  
*Chemotherapeutic activity of compound # 863 against the filarial parasite, L. carinii, of the cotton rat following intravenous administration*

DOSAGE INTERVAL	NO. OF DOSES	INTERVAL TO AUTOPSY	SINGLE DOSE  mgm./kgm.	INCIDENCE OF CURE	INCIDENCE OF FATALITY
24 hr.	3	96 hr.	0.5	2/3	
			0.75	0/3	
			1.0	3/4	
			10.0		2/2
	6	7 days	1.0	10/10	
			7.5		0/4
			10.0		2/10
72 hr.	3	96 hr.	1.0	6/7	
			10.0		0/2
			12.5		3/3
	4	7 days	1.0	3/6	
			1.25	10/10	
			10.0		0/5
	6	7 days	12.5		3/5
			1.0	11/11	
			10.0		1/10
7 days	3	7 days	1.0	13/15	
			10.0		0/10
			12/5		3/4
			15.0		1/3
	6	7 days	1.0	8/9	
			10.0		1/10
	1		12.5		0/10
			15.0		4/10
			17.5		2/5

that an increase in the size of the total dose, rather than that of the individual injections, was the critical factor in this increase in the frequency of cures, it should have been possible to effect a similar increase with the usual dose of 1.0 mgm. per kgm. merely by increasing the number of injections to five or more. Therefore, a group of animals was given six doses each of 1.0 mgm. per kgm. of # 863 intravenously, at intervals of either 24 or 72 hours, or at intervals of seven days. When autopsies were performed, seven days after the last dose, all of ten animals injected daily, and all of eleven animals injected at intervals of 72 hours

yielded only dead worms; in only one infested cotton rat of a total of nine were a few living worms found when the dosage interval was seven days.

The cumulative relationship between dosage and effect on the parasitic worms did not appear to be duplicated with regard to the effect on the host. Unless a given dose exceeded that which was acutely toxic, the cumulative effects of the drug were not striking. Thus 10 mgm. of #863 per kgm., given intravenously, caused a lethal effect in four of twelve animals when given daily (table 3); when this dosage was given repeatedly at intervals of 72 hours, or longer, only two of 37 animals were killed by the same treatment (table 3). The findings suggest that very little toxic effect was carried over from one dose to the next. This is supported also by the fact that the minimum dose which caused death following a single injection (15.0 mgm. per kgm.) was only slightly greater than that (12.5 mgm. per kgm.) which frequently was lethal on repeated administration.

On the basis of these experiments, tentative plans were made for the use of similar treatment schedules in the preliminary clinical trials of #863, that is to say, schedules in which repeated intravenous injections would be given at intervals sufficiently long to obviate or to minimize the danger of cumulative toxic reactions. These clinical trials, performed with patients harboring the filarial worm, *Wuchereria bancrofti*, were preceded by extensive studies of the effects of repeated intravenous injections of #863 in dogs and monkeys. Both the clinical trials, which unfortunately were unsuccessful, and the toxicity studies just mentioned, will be described in future publications (4, 7).

*D. The effect of #863 on the microfilaremia of cotton rats infested with L. carinii.* In infested cotton rats the effectiveness of an antifilarial agent may be determined directly by examination of the worms removed from the pleural cavities of treated animals at autopsy. However, in human patients harboring *W. bancrofti* in the lymphatic system, indirect criteria for evaluation of the effectiveness of such an agent unfortunately cannot be avoided. Periodic counts of the microfilariae in the peripheral blood have been included by most investigators as one procedure in following treated patients, since a decrease in such counts may reflect the death of adult worms (8, 9). With drugs not lethal to the larvae themselves, such a reduction should occur at a slow rate, over an interval of time related to the longevity of the microfilariae present at the time when their continued production is curtailed by the death of, or injury to, the adult worms. A rapid reduction in, or disappearance of, the microfilaremia would suggest a direct effect of the medication on these larvae, unrelated to the effect on the adult parasites, and would necessitate the use of other indirect criteria in the appraisal of the therapeutic result. For this reason it appeared of interest to follow the microfilaremia of cotton rats harboring *L. carinii* after treatment with #863.

After performing several counts of the microfilariae in the blood of seven rats, five of the animals were given 0.5 mgm. of #863 per kgm. intraperitoneally once daily for five days; the remaining two rats served as untreated controls. The counts were repeated at various intervals over a period of six months.

The counts were performed as follows: 20 cu.mm. of blood were withdrawn from the tail into a hemoglobin pipette previously moistened with heparin solution, and were spread on

a glass slide over an area of approximately 3 cm. x 1 cm. When dry, this smear was covered with a 1 per cent solution of gentian violet in 1 per cent acetic acid and the slide was rocked gently until all the blood had been laked, following which a cover glass was placed over the wet preparation. All the microfilariae on the slide were counted under the low power of the microscope, a process facilitated by the staining of the larvae with gentian violet.

The results of this experiment are recorded in table 4. The drug had no immediate pronounced effect on the degree of microfilaremia. On the other hand a slowly progressive reduction in the number of circulating microfilariae occurred; after five months the count reached zero. The number of microfilariae in the blood of the untreated control cotton rats did not decrease during the experimental period; in fact, in one case, a marked increase occurred. At autopsy, the treated rats yielded only dead worms which were degenerating and massed in exudate. One control rat escaped before the end of the experiment; the other one, at autopsy, yielded only actively motile worms showing no gross abnormalities. These results indicate that the gradual decrease in the microfilaremia of the cotton rat following therapy with the cyanines was due to the death of the adult worms. Even if a direct delayed action on the larvae occurred, it would be difficult to explain a delay so great as to allow survival of some larvae for many months after the drug surely must have been entirely excreted or destroyed.

*E. The effect of cyanine dyes on the dog heart worm, Dirofilaria immitis.* In addition to #863, several other cyanines listed in our first paper (1) were tested for chemotherapeutic action against this filariid; they were #348, #715, #963, #964, and #965. An entirely different sequence of events occurred in this situation than in that involving *L. carinii* and the cotton rat.

Following the repeated administration of any one of the dyes used, intravenously, a rapid diminution in the microfilaremia occurred, suggesting a direct action on the larvae. The initial counts were of a much lower order of magnitude than were those in the cotton rats (e.g., a maximum of 200 per 20 cu. mm. of blood). These began to decrease immediately following the initiation of therapy, reaching levels of less than one microfilaria per 20 cu. mm. of blood within several days. Such low counts persisted even with dogs followed for 3½ weeks after the completion of treatment, only an occasional larva being found on repeated thick smears, or following concentration from large blood samples by hemolysis and centrifugation. On the other hand, even in dogs which received repeated injections of maximally tolerated doses of #863, autopsies performed within a 3½ week period after therapy showed that all the adult worms removed from the heart and great vessels were alive and actively motile. Microscopic examination of histological sections of some of these worms revealed the presence of pathological changes in the reproductive system of the females sufficient in extent to curtail the production of further microfilariae and their discharge into the blood stream.<sup>5</sup> Similar changes produced by the antimonials have been described by Ashburn *et al.* (10) who state that "longer periods of observation would be necessary to

<sup>5</sup> We express our sincere thanks to Dr. L. L. Ashburn and his coworkers (10) of the Pathology and Zoology Laboratories, National Institute of Health, Bethesda, Maryland, who prepared, examined, and interpreted the histological sections of *D. immitis*.

TABLE I  
*The effect of cyanine dye #863 on the microfilaremia of cotton rats infested with L. carinii*  
 Dosage: 0.5 mgm. per kgm., administered intraperitoneally once daily on 5 successive days

RAT #	MICROFILARIAE PER 20 CU MM. OF BLOOD																			
	Days before Therapy					Days after Therapy														
	5	3	1	2	9	13	20	30	40	50	60	70	80	90	115	135	155	190	200	
1	2991	3889	—	2806	1802	1832	1378	960	522	418	110	186	123	55	1	2	1	—	—	
2	1519	1945	—	2581	802	1861	1772	1690	1396	1248	618	280	392	215	71	48	29	0	0*	
3	1982	2551	3316	3005	1828	2212	1912	2090	1690	1450	1124	516	518	333	104	61	1	0	0*	
4	1780	1737	—	1310	1132	1275	1258	978	918	550	384	350	150	Dead	—	—	—	—	—	
5	2230	3243	—	3131	2339	2673	2280	2086	2040	1854	412	360	347	224	51	77	37	0	0*	
Control	2094	—	—	2544	—	2991	3903	4163	—	—	4995	—	—	3213	2482	2532	772	—	9042†	
Control	3486	—	—	5356	—	6027	8877	10,173	—	—	8280	—	—	5112	6012	8216	—	—	—	

\* Masses of exudate, containing only dead worms in various stages of degeneration, found at autopsy.

† Twenty discrete, actively motile worms found at autopsy.

determine whether or not the sterilization is permanent." Similarly, it is not inconceivable that our animals might have shown a recurrence of microfilaremia if autopsy had been delayed for a longer period of time.

If a complete curtailment of the production of microfilariae, due to sterilization of the female worms, had been the only action of cyanines against *D. immitis*, the diminution in the number of circulating microfilariae should have been a gradual one, occurring over an interval proportional to the life span of the larvae present at the time of treatment. The total life span of these organisms is known to be a matter of months (8); yet the microfilariae disappeared from the blood following cyanine therapy in the space of only a few days. Therefore, a direct lethal action of the drug on the existing microfilariae must have occurred, curtailed production serving as an explanation only for the failure of killed larvae to be replaced within a short time after therapy.

Had sterilization of the female filariae been the only effect of therapy, the resultant slow decrease in the number of circulating microfilariae, and their failure to be replaced at a rapid rate, might have invited the false conclusion that all the adult worms were dead. This serves to re-emphasize a warning mentioned by other investigators (e.g., Brown, 8), that in the case of human filariasis, where a direct check on such a conclusion is not possible, extreme caution should attend the interpretation of decreases in microfilariae counts.

These experiments with *D. immitis* demonstrated that the marked chemotherapeutic action of the cyanines against *L. carinii* need not extend to all species of filariae. Unfortunately, this was borne out further by the finding that #863 was ineffective against *W. bancrofti* in man (7).

#### SUMMARY

(1) Twelve cyanine dyes, reported in a previous paper (1) to be the most active of a larger series against the filarial parasite, *L. carinii*, of the cotton rat, were studied further with regard to their efficacy by various routes of administration and to their relative suitability for clinical trial in human filariases.

(2) In contrast to the marked chemotherapeutic activity exerted by these compounds following intraperitoneal administration to infested cotton rats, oral administration even of massive doses resulted in only occasional cures.

(3) Cures were produced following subcutaneous administration, but the therapeutic indices were less favorable than those obtained when the intraperitoneal route was used. Furthermore, the compounds produced, at the sites of injection, local tissue injury of a sufficient degree to preclude their administration by this route to human patients.

(4) One of the intrinsically most active compounds, 1'-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2'-cyanine chloride, was selected for further study using the intravenous route of administration, since it produced less circulatory depression and less local tissue injury than any of the other compounds studied.

(5) This compound was found capable of producing cures in infested cotton rats when six doses each of 1.0 mgm. per kgm. were administered intravenously at either 24-hour, 72-hour, or weekly intervals. Lethal results followed the intra-

venous administration of single doses of 15 mgm. per kgm., and some animals were killed by the repeated injection of 10 mgm. per kgm., at intervals of one day. The wide range of dosage intervals over which the therapy was successful is discussed and the conclusion is drawn that the chemotherapeutic effect of the drug is related more directly, within limits, to the total amount of drug administered than it is to the frequency of administration.

(6) The course of the microfilaremia following administration of cyanines to cotton rats infested with *L. carinii*, and to dogs infested with *D. immitis* is described, as is the failure of these dyes to kill adult worms in the latter case. The implications for the clinical trial of #863 in patients harboring *W. bancrofti* are discussed.

#### REFERENCES

1. PETERS, L., BUEDING, E., VALK, A. D., JR., HIGASHI, A., AND WELCH, A. D.: THIS JOURNAL, 95: 212, 1949.
2. BUEDING, E.: J. Exper. Med., 89: 107, 1949.
3. WRIGHT, H. N., LITCHFIELD, J. T., JR., BRET, T., CRANSTON, E. M., CUCKLER, A. C., AND BIETER, R. N.: Annals N. Y. Acad. Sciences, 60: 109, 1948.
4. PETERS, L. *et al.*: To be published.
5. EAGLE, H., AND HOGAN, R. B.: Ven. Dis. Inf., 24: 33, 1943.
6. SWINYARD, E. A., HIRSCHFELDER, A. D., AND WRIGHT, H. N.: THIS JOURNAL, 75: 367, 1942.
7. SANTIAGO-STEPHENSON, D., WELCH, A. D., OLIVER-GONZALEZ, J., AND PETERS, L.: To be published.
8. BROWN, H. W.: J. A. M. A., 126: 952, 1944.
9. CULBERTSON, J. T., ROSE, H. M., AND OLIVER-GONZALEZ, J.: Am. J. Trop. Med., 25: 271, 1945.
10. ASHDURN, L. L., FERRIN, T. L., BRADY, F. J., AND LAWTON, A. H.: Arch. Path., 40: 334, 1945.
11. PETERS, L., AND HIGASHI, A.: Fed. Proc., 8: 324, 1949.



# THE EFFECT OF CHOLINESTERASE INHIBITORS ON THE TOXICITY OF PROCAINE IN MICE<sup>1</sup>, <sup>2</sup>

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The existence in blood of an enzyme catalyzing the hydrolysis of procaine to *p*-aminobenzoic acid and diethylaminoethanol was first reported by Legge and Durie in 1942 (1). Since then many workers have confirmed this finding and have studied the procainesterase activity in human and animal sera (2-6). It has also been demonstrated that procainesterase can be inhibited *in vitro* by anticholinesterase agents such as fluoride, methylene blue, neostigmine, and physostigmine (7). Kisch *et al.* (5, 8, 9) showed, however, that this enzyme is distinct from cholinesterase since the procainesterase activity does not run parallel to the cholinesterase activity in different species of animals, and the former activity resides almost wholly in the albumin fraction of the serum. The extent of the influence which such inhibitors of procainesterase might have on the hydrolysis rate of procaine *in vivo* remains unsettled. Burgen and Keele reported in 1948 (10), that methylene blue, neostigmine, physostigmine, and di-isopropyl fluorophosphate did not significantly affect procaine blood levels attained from intravenous infusion of procaine in chloralosed cats.

If procainesterase is an important factor in the metabolism of procaine *in vivo* it would be anticipated that drugs which inhibit this enzyme would increase the toxicity of procaine. The experiments described in the present paper were designed to determine whether the agents which have been shown to exert an antiprocaesterase action *in vitro* would affect the toxicity of procaine in mice. Since these drugs also have well established anticholinesterase action other drugs having this latter action were tested as well.

**PROCEDURE.** Healthy white mice of both sexes, weighing 20 to 23 gm. each, were injected subcutaneously with the drug to be tested for its influence on procaine toxicity. Premedication doses were selected as the maximum amounts that would not be responsible, in themselves, for any deaths. The solvent, concentration, and dose used for each drug are indicated in table 1. After a time interval estimated to be needed by the drug to attain its maximum action—ten minutes for each drug except DFP which was dissolved in peanut oil and required an estimated four to five hours for maximum action—1 per cent procaine hydrochloride in saline was injected rapidly into the tail vein. Different groups of animals premedicated with a given drug received doses of procaine chosen in an attempt to span its expected LD<sub>50</sub>. By the method of Reed and Muench (11), the observed mortality percentage was converted to calculated percentage. From these a graphic solution of the dosage-effect curve was made on probit paper according to Litchfield and Fertig (12). The LD<sub>50</sub> in each case was then that value on the abscissa for which the ordinate was five.

Five drugs were tested in this way for their effect on procaine toxicity: physostigmine

<sup>1</sup> Preliminary report, Fed. Proc., 7: 212, 1948.

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salicylate; neostigmine methyl sulfate; diisopropyl fluorophosphate (DFP); carbamic acid, N,N-dimethyl-4-dimethylamino-3-isopropylphenyl ester, methiodide (DIP) (13); and methacholine chloride. Environmental temperature varied less than one degree centigrade during these experiments.

**RESULTS AND DISCUSSION.** The results of experiments to ascertain the influence of various cholinesterase inhibitors and of methacholine on procaine mortality in mice are shown in table 1. In the mice receiving no premedication, the LD<sub>50</sub> of intravenous procaine was 53.7 mgm./kgm.

When physostigmine salicylate premedication was given ten minutes before procaine, the LD<sub>50</sub> of the latter was 66.1 mgm./kgm. This seemed an anomalous

TABLE 1

*Toxicity of intravenous procaine in mice previously treated by subcutaneous injection of various anticholinesterase agents*

DOSE PROCAINE MGM./KGM. I.V.	PREMEDICATION AND MORTALITIES					
	None	Physostigmine salicylate, 0.01% in saline 0.3 mgm./kgm	Neostigmine methyl-SO <sub>4</sub> , 0.01% in saline 0.15 mgm./kgm.	DFP 0.01% in peanut oil 2 mgm./kgm.	DIP* 0.001% in saline 49 microgm./ kgm.	Methacholine- Cl 0.1% in saline 10 mgm./kgm.
70	28/20	15/25	—	5/6	—	—
65	—	10/25	—	17/32	—	—
60	51/75	7/25	4/4	12/28	—	—
55	22/41	—	—	3/10	—	17/26
50	22/51	0/10	10/10	—	14/19	9/25
45	3/26	—	—	—	—	3/20
40	1/31	—	14/20	—	11/19	—
35	—	—	11/20	—	2/19	—
30	0/10	—	5/20	—	—	—
LD <sub>50</sub> ± S.E.	53.7 ± 0.5	66.1 ± 0.1	34.9 ± 0.9	62.4 ± 0.8	41.9 ± 1.2	51.8 ± 0.8
t Value	—	10.1	15.3	8.4	9.2	2.0

\*Carbamic acid, N,N-dimethyl-4-dimethylamino-3-isopropylphenyl ester, methiodide.

Note: Physostigmine, neostigmine, and DIP were given 10 minutes before the procaine. The DFP in peanut oil was administered 4 to 5 hours before the procaine.

finding, as it demonstrated some protective action of this drug in spite of its reported *in vitro* antiprocainesterase activity. Following premedication by this drug a decrease was noted in the incidence and severity of the convulsions resulting from the intravenous procaine, as well as a more rapid recovery from its effects.

Neostigmine methylsulfate, administered ten minutes before procaine, yielded strikingly different results. The LD<sub>50</sub> of procaine in mice pretreated with this drug was 34.9 mgm./kgm. This increased toxicity of procaine was the effect anticipated if one assumed an inhibition of procainesterase by neostigmine. Deaths resulting were of the kind seen with the procaine controls, i.e., violent convulsions followed immediately by respiratory failure, while the heart continued to beat for some minutes thereafter.

Four to five hours were allowed to elapse after DFP before the intravenous injection of procaine was given. In this experiment the  $LD_{50}$  of procaine was 62.4 mgm./kgm. indicating that DFP, like physostigmine, offers some protection against the lethal action of the procaine. DFP and physostigmine have both been shown to gain ready access to brain cholinesterase *in vivo*, in contrast to neostigmine (13). This may be of some significance in explaining the differential action of these agents in their influence on procaine toxicity.

In the study using DIP, the procaine was injected ten minutes after the premedication. Here the  $LD_{50}$  of procaine was reduced to 41.9 mgm./kgm. These data in turn are comparable to those obtained from the use of neostigmine and correlate with the fact that DIP, like neostigmine, does not gain ready access to brain cholinesterase *in vivo* (13). DIP has not been shown to inhibit procainesterase *in vitro*.

Since the premedicants used thus far are parasympathetic stimulants by virtue of their inhibiting action on cholinesterase, it was desirable to test the effect upon procaine toxicity of a parasympathetic stimulant without anticholinesterase effect. Methacholine was chosen for this experiment. This parasympathomimetic drug was injected subcutaneously as the chloride salt in a 0.1 per cent concentration in normal saline ten minutes prior to the procaine. The  $LD_{50}$  of procaine under these conditions was 51.8 mgm./kgm. This was not considered significantly different from the control value and was taken to indicate that the changes in toxicity of procaine induced by the anticholinesterase agents are probably not a function of an altered metabolism of acetylcholine and are probably not secondary to the parasympathetic actions of these drugs.

The data presented above have shown that the cholinesterase inhibitors tested exert significant but inconsistent influences upon the toxicity of intravenous procaine in the mouse. Physostigmine and DFP offered some protection against the lethal action of procaine, while neostigmine and DIP augmented its lethal action. Though the effects in either case were small they were nonetheless real and require some attempt at an explanation. The increase in procaine sensitivity of the mice treated with neostigmine and DIP might well be attributed to the inhibition by these drugs of the enzyme destroying procaine. The question then arises, however, why physostigmine and DFP, which have *in vitro* procainesterase inhibiting qualities, do not also increase the mouse sensitivity to procaine. One might suppose that the latter two drugs present a pharmacologic action which overbalances their antiprocainesterase effect to the extent that protection against procaine is the net result. There is little to indicate what the nature of such an action might be. It was pointed out, however, that mice premedicated with physostigmine evidenced less severe convulsions following the injection of procaine than did the untreated animals. Thus it appears possible that physostigmine presents an antagonistic action against the central effects of procaine. Physostigmine, in toxic doses, has been stated to have depressant effects on the brain and spinal cord (16).

A further difference between the actions of neostigmine and physostigmine is the fact that the former drug exerts a direct cholinergic effect upon skeletal muscle

independent of its anticholinesterase property (17). This action of neostigmine could be thought to augment the convulsive action of procaine and hence increase its toxicity. In the light of this possibility, however, it is not understood why methacholine did not also increase the toxicity of procaine. The different mechanisms of action of physostigmine and neostigmine are further reflected by the fact that the former drug protects animals against the toxic effect of DFP (18) and of parathion (19), two potent cholinesterase inhibitors, while the latter drug offers much less protection (20). A possible explanation of this difference is that physostigmine combines more readily than does neostigmine with brain cholinesterase *in vivo* (13), thus protecting it from combination with subsequently administered esterase inhibitors.

An interesting correlation, but one which throws little light upon the question, is the fact that physostigmine and DFP, which offered protection against procaine are fat soluble and inhibit brain cholinesterase *in vivo* (13) more readily than do neostigmine and DIP which are not fat soluble and have the opposite effect on procaine toxicity. On this basis, however, DFP would be expected to give better protection than physostigmine since the former gains readier access to brain esterase (13). Though the reverse was true in our experiments it is possible that the DFP had not attained its maximum effect.

The above discussion leaves open the question of the mechanism by which the premedicating agents used influence the toxicity of procaine. In any case it appears unlikely that changes in the rate of enzymatic breakdown of procaine played an important role in determining the effects observed. However this possibility can be eliminated only by a study of the rate of disappearance of procaine from mouse blood *in vivo* and from the whole mouse following intravenous injection of procaine.

#### SUMMARY

1. Four cholinesterase inhibitors and one non-esterase inhibiting parasympathetic stimulant were tested for their effect on intravenous procaine toxicity in mice premedicated with the drugs.

2. Physostigmine and DFP showed significant protective effects, increasing the  $LD_{50}$  of procaine 23 and 16 per cent, respectively.

3. Neostigmine and carbamic acid, *N,N*-dimethyl-4-dimethylamino-3-isopropylphenyl ester, methiodide increased the susceptibility of mice to the toxic action of intravenous procaine, lowering the  $LD_{50}$  35 and 22 per cent, respectively.

4. Methacholine had no statistically significant effect on the toxicity of procaine.

5. These results indicate that the reputed antiprocinesterase activity of the cholinesterase inhibitors probably does not influence the toxicity of intravenous procaine in mice, and that other factors must be considered to explain the influence which these agents were seen to exert on procaine toxicity.

#### REFERENCES

1. LEGG, J., AND DURIE, E.: *M. J. Australia*, 2: 561, 1942.
2. GOLDBERG, A., KOSTER, H., AND WARSHAW, R.: *Arch. Surg.*, 46: 49, 1943.

3. HAZARD, R., AND RAVASSE, J.: Bull de L'Acad. de Med., 129: 535, 1945.
4. KISCH, B., AND STRAUSS, E.: Exper. Med. and Surg., 1: 367, 1943.
5. KISCH, B., KOSTER, H., AND STRAUSS, E.: Exper. Med. and Surg., 1: 51, 1943.
6. KISCH, B., AND KOSTER, H.: Exper. Med. and Surg., 1: 71, 1943.
7. KISCH, B.: Exper. Med. and Surg., 1: 84, 1943.
8. STRAUSS, E., AND KISCH, B.: Exper. Med. and Surg., 1: 371, 1943.
9. KISCH, B.: Exper. Med. and Surg., 1: 278, 1943.
10. BURGÉN, A. S. V., AND KEELE, C. A.: Brit. J. Pharmacol., 3: 128, 1948.
11. REED, L., AND MUENCH, H.: Am. J. Hyg., 27: 493, 1938.
12. LITCHFIELD, JR., J., AND FERTIG, J.: Bull. Johns Hopkins Hosp., 69: 276, 1941.
13. DuBOIS, K. P., ERWAY, W. F., AND BYERRUM, R. U.: Fed. Proc., 6: 326, 1947.
14. BRATTON, A., AND MARSHALL, JR., E. K.: J. Biol. Chem., 128: 539, 1939.
15. FORNEY, R.: Personal Communication, 1948.
16. GOODMAN, L., AND GILMAN, A.: *The Pharmacological Basis of Therapeutics*, New York, MacMillan, 1941, p. 380.
17. RIKER, W. F., AND WESCOE, W. C.: THIS JOURNAL, 88: 58, 1946.
18. KOSTER, R.: THIS JOURNAL, 88: 39, 1946.
19. DuBOIS, K. P., DOULL, J., SALERNO, P. R., AND COON, J. M.: THIS JOURNAL, 95: 79, 1949.
20. SALERNO, P. R., AND COON, J. M.: Unpublished data.

## THE ABSORPTION, DISTRIBUTION AND EXCRETION OF ISONIPECAINE (DEMEROL)

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Due to the development of a rapid sensitive chemical technique by Brodie *et al* (1) for analyzing basic amines, it is now possible to follow the absorption, distribution, fate and excretion of many compounds of therapeutic value. We have been able to apply the Brodie methyl orange method with but slight modification for determining isonipecaine (Demerol, meperidine) in biological tissue and consequently were able to demonstrate *in vitro* and *in vivo* that the liver is essential for the detoxication of the compound (2). In continuation of our studies on the fate of isonipecaine, we are presenting findings on its pharmacologic distribution and excretion.

**PROCEDURE AND RESULTS** *Determination of isonipecaine and examination of specificity*  
Inasmuch as the methyl orange method is applicable for most basic amines, it is necessary to appraise the specificity for isonipecaine. According to Brodie (1) this can be accomplished by determining the distribution of a drug in an organic solvent water system at various pH values of the aqueous phase. The probability that two different compounds may have the same distribution coefficients under such physically defined conditions is rather remote. Accordingly, the experimental procedure to examine specificity of the method for isonipecaine recovered from biologic tissue was carried out in the following manner. Several rats were given a total of 100 mgm /kgm of isonipecaine intraperitoneally in two divided doses over a two hour period. Thirty to sixty minutes after the last injection the animals were sacrificed. Various organs were removed, like ones pooled, diluted 1:15 in M/50 sodium fluoride and then minced thoroughly in a Waring Blendor. After addition of sufficient dilute alkali, each tissue mince was extracted with twice its respective volume of ethylene dichloride. The ethylene dichloride was washed thrice with one half its volume of M/2 phosphate buffer pH 7. The buffer layer was completely removed by centrifugation and aspiration. Two 7.5 cc samples of the ethylene dichloride extract were reacted with methyl orange and analyzed for isonipecaine content. Other 7.5 cc samples of ethylene dichloride were each shaken thoroughly with an equal volume of M/2 buffer solutions of pH 7, 6 and 5 and then analyzed for isonipecaine after removal of the buffer layer. The results were compared with those obtained from similar treatment of an aqueous solution of isonipecaine and from isonipecaine added to various biologic specimens.

The results are listed in table 1. It is apparent from a consideration of the data that the various tissue extracts, especially the liver, contain methyl orange-reacting material with solubility characteristics that differ from those of pure isonipecaine. A small amount is due to tissue blanks, but the main contribution is presumably from isonipecaine metabolites. By introducing a triple buffer wash

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to the method, however, practically all the metabolic products of isonipecaine, plus blank substances in tissue which react with methyl orange, are excluded and a high degree of specificity of the method for isonipecaine is obtained. In the case of the liver even three buffer washings are not always sufficient to remove all the isonipecaine metabolites. However, after the triple wash usually over 85 per cent of the methyl orange reactants in the liver represents isonipecaine. These findings are in line with Brodie's observation that as a general rule basic amines are metabolized in the body to substances more water-soluble than the parent compounds.

TABLE 1

*Distribution ratios\* of added and recovered isonipecaine between water and ethylene dichloride after equilibration with various buffers*

SAMPLE OF ISONIPECAINE	NO. OF BUFFER WASHINGS	pH		
		7	6	5
Added to water.....	1	0.97	0.77	0.35
<i>Rat tissues</i>				
Added to liver.....	1	0.99	0.73	0.38
Recovered from liver.....	1	0.61	0.46	0.20
Recovered from liver.....	3	0.89	0.60	0.33
Recovered from brain.....	1	0.87	0.54	0.26
Recovered from brain.....	3	0.97	0.80	0.33
Recovered from lung.....	3	0.93	0.79	0.37
Recovered from heart.....	3	1.00	0.80	0.39
Recovered from spleen.....	3	0.96	0.80	0.33
Recovered from kidney.....	3	0.93	0.77	0.34
Recovered from muscle.....	3	1.02	0.75	0.40
Added to blood.....	1	0.94	0.77	0.39

\* concentration remaining in ethylene dichloride

concentration in ethylene dichloride before equilibration

*Rate of absorption of isonipecaine in rats.* To rule out possible destruction of isonipecaine by the gastro-intestinal tract, two rats were given a known amount of the compound by stomach tube and sacrificed immediately. Their entire gastro-intestinal tracts plus contents were removed, suspended in isotonic saline, and incubated for two hours; the samples were then minced and analyzed for isonipecaine content in the usual manner. No change in isonipecaine concentration was noted after incubating.

Isonipecaine was then administered by stomach tube to a group of nonfasted rats and to another group fasted for twenty hours. The amount of isonipecaine remaining in the gastro-intestinal tract was estimated one, two and four hours later.

Rapid absorption of isonipecaine is suggested by the fact that only a small fraction of the compound was recovered from the gastro-intestinal tract after only one hour, figure 1. After four hours less than 10 per cent of the total administered isonipecaine was found. It is possible that excretion by this route does occur, but the findings suggest that this factor is relatively minor. It appears also that the presence of food in the stomach slows absorption of isonipecaine. This is in line with our earlier studies where we reported that nonfasted rabbits survived an oral dose of isonipecaine usually lethal for fasted rabbits (3).

*Distribution of isonipecaine.* The distribution of isonipecaine was studied in several rats and one dog. After administration of the drug, the animals were sacrificed at various time intervals. One gram (whenever possible) of blood, heart, liver, kidney, brain, spleen, muscle and lung was removed and suspended in sufficient *M/50* sodium fluoride to make 15 cc. and then minced in a Waring Blendor. A 5 cc. aliquot was analyzed for isonipecaine content in the usual manner (2) with the exception that a triple buffer wash was used instead of a single wash.

The results in table 2 indicate that isonipecaine tends to concentrate in the kidneys, spleen, lungs and liver. Appreciable levels are attained in the brain and somewhat lower concentrations in the heart and muscle. Very little isonipecaine was found to be present in blood.

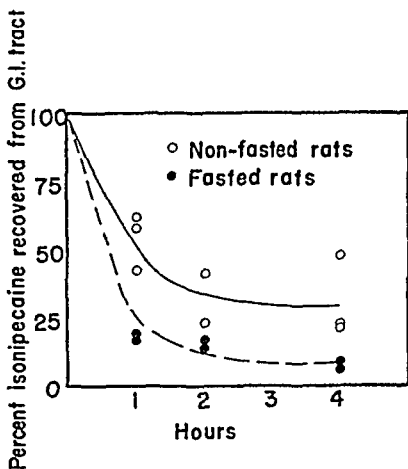


FIGURE 1. Rate of disappearance of isonipecaine from the gastro-intestinal tract in rats after 50 mgm./kgm. orally.

Evidence that storage of isonipecaine does not extend over a great period of time is indicated by the fact that tissue concentrations were barely detectable five hours after administration of 100 mgm./kgm. isonipecaine (table 2). Also even after nine successive injections of isonipecaine (50 mgm./kgm.) over a three-day period, little or no isonipecaine was detected in the animals sacrificed on the fourth day. Since it is well known isonipecaine has a relatively short duration of action and, in view of our previous report, that the liver rapidly metabolizes isonipecaine, the results obtained are somewhat to be expected.

The results obtained on one dog after administration of isonipecaine followed the same distribution pattern as in the rats, with the exception that a low level was found in the lungs. Highest levels were found in the kidney, liver, and spleen.



*Excretion of isonipecaine in saliva.* Eight patients were given 100 mgm. of isonipecaine intramuscularly and were instructed to collect their saliva for the first and second hours. The samples were analyzed for isonipecaine content. Control studies on saliva obtained prior to drug administration from six volunteers who received no isonipecaine indicated that negligible methyl orange reactants were present.

Low concentrations of isonipecaine varying from 3.5 mgm./liter to 6.0 mgm./liter were found excreted in the saliva. Due to the small amounts detected, it was not feasible to evaluate for the specificity of the method. It is possible, therefore, that isonipecaine metabolite(s) might have contributed to a small degree to the obtained values, in which case the values would be even lower than those now given.

TABLE 2

*Concentration of isonipecaine (mgm./kgm. fresh tissue) in rats after intraperitoneal administration*

DOSAGE SCHEDULE	BLOOD	LUNG	LIVER	KIDNEY	SPLEEN	BRAIN	HEART	MUSCLE
0 hr. 50 mgm./kgm. 1 hr. 50 mgm./kgm. 2 hrs. sacrificed	3*	11*	34*	46*	58*	17*	10*	7*
0 hr. 50 mgm./kgm. 1 hr. 50 mgm./kgm. 3 hrs. sacrificed	2 3 4 5 <2	20 28 20 14 19	16 19 14 11 14	110 57 43 22 20	21 27 60 16 29	10 8 23 14 12	6 15 8 7 10	5 4 8 4 4
0 hr. 50 mgm./kgm. 1 hr. 50 mgm./kgm. 5 hrs. sacrificed	<2 <2	12 —	12 18	16 4	3 <2	4 —	<2 <2	4 <2
50 mgm./kgm. 3 times daily for 3 days. Sacrificed on 4th day.	<2 <2	<2 <2	<2 <2	<2 <2	3 <2	<2 <2	<2 <2	<2 <2

\* Average values of pooled organs of 5 rats.

*Excretion of isonipecaine in milk.* Six lactating patients were given 100 mgm. of isonipecaine by the intramuscular route and the milk was collected at various time intervals between one and six hours after injection. On analysis for isonipecaine no significant amount was detected, the concentration being less than 0.5 mgm./liter. No difficulty was encountered recovering isonipecaine added to human milk.

*Placental permeability to isonipecaine.* Evidence that isonipecaine crosses the placental barrier is indicated by the following experiment. Isonipecaine (100 mgm. I.M.) was administered once or twice to expectant mothers during the first stage of labor. Total urine specimens of their respective newborns were collected for the first 24 hours and estimated for isonipecaine content. Only males were used. A urine sample was obtained with very little loss by placing a rubber condom over the base of the penis immediately after delivery.

To prevent spilling and to effect a snug fit, a Kelly clamp was then used to close the open end of the condom.

The results are shown in table 3. Varying concentrations of isonipecaine were found in the urine, whereas five control specimens, collected from infants whose mother received no isonipecaine, showed no appreciable levels of methyl orange-reactants (less than 1 mgm./liter). That the isonipecaine found is chiefly the com-

TABLE 3

*Amount of isonipecaine in urine excreted by mothers and their respective newborns 24 hours after administration of the compound*

PATIENT	CONCENTRATION MCGM./L.		% TOTAL DOSE	
	Son	Mother	Son	Mother
M	16	35	0.2	8.7
B	2	15	0.0	4.5
H	1	12	0.0	10.0
L	10	—	0.2	—
CO	18	—	0.6	—
W	8	—	0.1	—
He	6	—	0.0	—
O	4	—	0.0	—
ML	10	—	0.1	—

TABLE 4

*Distribution ratios\* of added and recovered isonipecaine between water and ethylene dichloride after equilibration with various buffers*

SAMPLE OF ISONIPECAINE	NO OF BUFFER WASHINGS	pH			
		14	7	6	5
Added to water	1	1.00	0.97	0.77	0.35
Added to adult urine	1	1.01	0.94	0.73	0.33
Added to adult urine	3	0.99	1.01	0.76	0.36
Recovered from adult urine	0	1.02	0.65	0.42	0.18
Recovered from adult urine	3	0.99	1.00	0.80	0.36
Recovered from newborn urine	1	—	0.67	0.52	0.22
Recovered from newborn urine	3	—	0.91	0.73	0.34

\*  $\frac{\text{concentration remaining in ethylene dichloride}}{\text{concentration in ethylene dichloride before equilibration}}$

pound itself is indicated in the specificity studies of table 4. The amount of isonipecaine which appeared in the newborn urine, however, was quite low. Less than 1 per cent of the total administered dose was accounted for by this route. This appears to support the clinical reports that isonipecaine usually has relatively minor adverse effects on the fetus when it is used to produce obstetrical analgesia.

*Excretion of isonipecaine in human urine.* Although the urinary excretion of isonipecaine has been studied by Lehman and Aitken (4) and by Oberst (5), no

evidence was presented as to specificity of method. Since we were able to determine isonipecaine in urine with a measurable degree of specificity, it was decided to investigate the urinary excretion of the compound.

Three patients were each given 100 mgm. of isonipecaine by the intramuscular route. Another individual took the same dose orally. Total urine specimens were collected at certain time intervals and analyzed for isonipecaine.

Less than 10 per cent of the total administered isonipecaine dosage was found to be excreted in the urine (figure 2). If due allowance is made for absorption of the drug, it can be readily seen that the rate of excretion is most rapid soon after administration of the compound. Single urine specimens from twenty or more individuals receiving isonipecaine obtained at various time intervals yielded values in line with the curves in figure 2. The amount of isonipecaine excreted

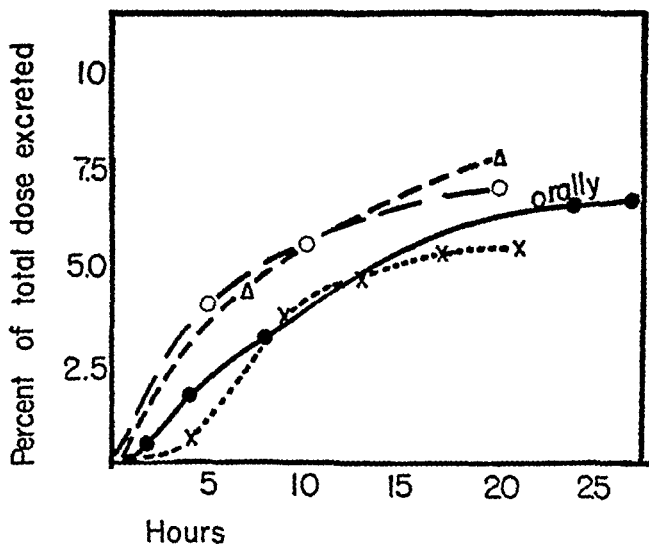


FIGURE 2. Rate of urinary excretion of isonipecaine in humans after intramuscular injection of 100 mgm.

ranged from 2-10 per cent of the total dose given. These findings are lower, but do not differ greatly from those reported by Lehman (4) and Oberst (5).

*Countercurrent studies on urines of individuals given isonipecaine.* In an attempt to fractionate and characterize the urinary metabolites and to obtain more rigid proof as to its presence in the urine in individuals receiving the drug, a modified Craig countercurrent technique (6, 7) was applied in the following manner:

Four individuals were given 100 mgm. isonipecaine orally before retiring. The urine was collected in the morning and analyzed for apparent isonipecaine content. About 4.5 per cent of the total administered dose was recovered as methyl orange reactants.

One hundred cc. of the urine were alkalized and extracted with small portions of ethylene dichloride until the urine gave negligible tests for methyl orange reactants. The ethylene dichloride was concentrated under reduced pressure to 30 cc. and a 25 cc. aliquot sample was employed for countercurrent distribution as described previously (8). An eight plate transfer separation was carried out in a system consisting of equal parts ethylene dichloride and 2M phosphate buffer pH 5.45.

At the end of the distribution the layers were separated and analyzed for methyl orange reactants. The values obtained were used for calculating the respective partition coefficients

of the methyl orange reactants in each bottle as well as to chart the characteristic distribution curve of the methyl orange reactants. The latter was obtained by plotting the fraction present in each bottle against the bottle number. The theoretical values were calculated in the manner described by Williamson and Craig (9).

From the distribution pattern of the methyl orange reactants in the urine, the results indicated that eight transfers were not sufficient for complete separation of the constituents for characterization although peaks were obtained at bottles 5 and 8. The findings suggested that the substance contained in bottle 5 might be isonipecaine since it was calculated from the partition coefficient of isonipecaine (determined prior to the countercurrent run) that it would give a maximum value at bottle 5.

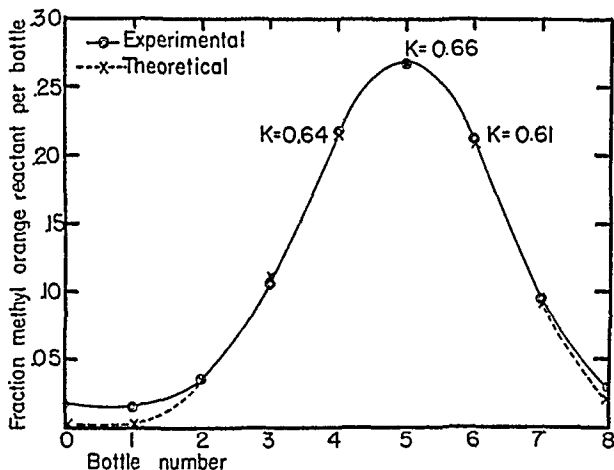


FIGURE 3. Countercurrent distribution curves of methyl orange reactants in urine of four individuals after taking 100 mgm. of isonipecaine orally.

In order to ascertain more precise information as to the identity of the isonipecaine-like substance in bottle 5, the countercurrent transfer of the same pooled urine of the four individuals was repeated, but the preparation of the urine sample for distribution was modified to exclude a great part of the methyl orange reactants other than isonipecaine. This was accomplished by using an adsorption technique previously used to separate methadone from blank substances in urine (10). Recovery of the isonipecaine from the adsorbent<sup>2</sup> was effected by alkalinizing and shaking with ethylene dichloride. Although blank substances and isonipecaine metabolites are carried through the process to some extent, considerable separation of isonipecaine from these substances can be effected by such means.

When the same urine sample of the above individuals was treated by the adsorption and elution technique and then subjected to an eight plate countercurrent transfer, the curve as shown in figure 3 was obtained. A substance with a maximum at bottle 5 and a partition coefficient of 0.66 yielded an experimental curve which fitted well with the calculated theoretical curve. When the partition

<sup>2</sup> Superfiltrol—Filtrol Corporation, Los Angeles, California.

coefficient of isonipecaine was determined for the same system as used in the countercurrent distribution, it was found to be 0.65. Thus, there is strong evidence indicating that the substance in bottle 5 is actually isonipecaine.

On further calculation, it was found that the isonipecaine present constituted 60 per cent of the total methyl orange reactants in the urine and represented less than 3 per cent of the total isonipecaine dosage. The remainder of the methyl orange reactants represented isonipecaine metabolite(s) and blank substances in the urine.

Countercurrent transfers on other urine samples of individuals who received isonipecaine indicated that the isonipecaine present varied from 25 to 75 per cent of the total methyl orange reactants. Since the amount of isonipecaine appearing in the urine usually represents less than 10 per cent of the administered dose, it is quite evident that urinary excretion plays a minor role in disposing of isonipecaine.

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#### SUMMARY

1. After administration of isonipecaine (Demerol) to rats the compound concentrates mainly in the kidneys, spleen, lungs and liver. Appreciable levels are attained in the brain and lower concentrations in heart and muscle.

2. After repeated administration of isonipecaine three times daily for several days virtually no isonipecaine could be recovered from the animals 24 hours later.

3. Isonipecaine is rapidly and almost totally absorbed after oral administration.

4. Isonipecaine or its metabolite(s) is excreted to some extent in saliva; but no isonipecaine was detected in the milk of individuals receiving the compound.

5. Less than 5 per cent of the total oral isonipecaine dosage was separated and characterized from urine by countercurrent techniques.

6. An even smaller amount of isonipecaine (less than one per cent) was found in the urine of newborns whose mothers had previously received isonipecaine during the first stage of delivery.

#### REFERENCES

1. BRODIE, B. B., AND UDENFRIEND, S.: *J. Biol. Chem.*, **158**: 705, 1945.
2. LEONG WAY, E., SWANSON, R., AND GIMBLE, A. I.: *THIS JOURNAL*, **91**: 178, 1947.
3. LEONG WAY, E.: *THIS JOURNAL*, **87**: 265, 1946.
4. LEHMAN, R. A., AND AITKEN, T.: *J. Lab. Clin. Med.*, **28**: 787, 1943.
5. OBERST, P. W.: *THIS JOURNAL*, **79**: 10, 1943.
6. CRAIG, L. C.: *J. Biol. Chem.*, **155**: 519, 1944.
7. CRAIG, L. C., COLUMBIE, C., MIGHTON, H., AND TITUS, E.: *J. Biol. Chem.*, **161**: 321, 1945.
8. LEONG WAY, E., SMITH, P. K., HOWIE, D. L., WEISS, R., AND SWANSON, R.: *THIS JOURNAL*, **93**: 368, 1948.
9. WILLIAMSON, B., AND CRAIG, L. C.: *J. Biol. Chem.*, **168**: 687, 1947.
10. CRONHEIM, G., AND WARE, P. A.: *THIS JOURNAL*, **92**: 98, 1948.

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